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Controlled Release Personal Use

Arthropod Repellent Formulation

Final [redacted] Report, Phase I

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BIOTEK, Inc.

21-C Olympia Avenue, Woburn, MA 01801 Tel: (617) 938-0938

AD_____

BIOTEK Report No. 2128-6

Controlled Release Personal Use Arthropod Repellent Formulation

Final Report, Phase I

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SEP 25 1986
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E.S. Nuwayser, Ph.D.
M.H. Gay, Ph.D.
D.J. DeRoo
J.A. Mahoney
P.D. Blaskovich

September 25, 1985

Supported by:

U. S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-84-C-4270

BIOTEK, Inc.
21-C Olympia Avenue
Woburn, MA 01801

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

BIOTEK, Inc. has developed an arthropod repellent formulation containing N,N,-diethyl-m-toluamide (Deet) for topical application to exposed skin which will provide protection for a duration of 12 hours. This has been achieved by formulating the active ingredient, Deet, with a proprietary blend of silica gel, a lotion base, and alcohol. The formulation is non-toxic, is cosmetically acceptable, is more compatible with military materials than the current in-use formulation, and complies with EPA specifications.

In vitro testing of our formulation in a Reifenrath permeation/evaporation cell using weanling pig skin shows an evaporation rate greater than the minimum effective evaporation rate described by the Request for Proposal for a period of 22 hours.

Under standard clinical test conditions of moderate temperature and humidity, the formulation provided 100% protection against Aedes aegypti mosquitoes for at least 12

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I. SUMMARY

In vitro evaporation and penetration rates of Deet and Deet sustained-release formulation were determined using the methods of Reifenrath and Robinson (1982).

Three types of silica gel, each with a different size and pore volume, were initially tested at 1:1 Deet:silica gel ratios. The matrices averaged a three-fold increase in total evaporated Deet compared to native Deet, while reducing percutaneous penetration by an average of sixty percent. When different weight ratios of these three Deet-silica gel matrices were tested on the *in vitro* apparatus, silica gel B-Deet matrices produced the most effective sustained release action, with a 1:1 Deet to silica gel weight ratio lasting the longest. Changing the substrate from hairless mouse skin to split thickness weanling pig skin and increasing the rate of air flow to 600 ml/min did alter the experimental values. However, the relationships among the different matrices were unchanged.

Silica gel B was compared to several modified and coated silica gels. Hydrophobic silica gel mixed with Deet clearly provided the longest effective action of any material tested. A 3:1 Deet to hydrophobic silica gel weight ratio was chosen for formulation into our final sustained release product.

An alcoholic lotion base was developed. The lotion is compatible with the Deet-silica matrix, does not detrimentally affect the sustained release action of the matrix, and enhances the cosmetic properties of the formulation.

Final Deet concentration in the prototype product, HSL-44, is 44% by weight. The evaporation rate of this repellent at a 2 mg/cm^2 dose with weanling pig skin substrate at 500 ml/min. of air was maintained above the minimum effective evaporation rate of 5 ug/cm^2/hr. for at least twenty-two hours.

Using male subjects of military age, several sustained-action arthropod repellent formulations were tested for efficacy against *Aedes aegypti* mosquitoes under a variety of climatic conditions. Three repellent formulations provided 100% protection against biting under moderate climatic conditions (24°C, 65% relative humidity) for a period of at least twelve hours. Under basic hot, variable high humidity and hot and humid conditions, protection was provided for between eight and twelve hours. Formulations HSL-44 and HSL-50 provided the longest duration of repellency. The major causes of reduced duration of action under these more adverse conditions was the heavy perspiration of the test subjects and possibly rubbing the test sites on the arms against the body.

The presence of acrylic camouflage face paint applied previous to the application of HSL-44 did not alter HSL-44 efficacy.

A group of 13 male and 12 female test subjects of military age judged the cosmetic acceptability of HSL-44. HSL-44 was rated as "liked slightly" or higher by 72% and rated as "neither like nor dislike" or higher by 89% of the test subjects. The mean score was 5.1 out of a possible 7. It was observed that 85% of the male panelists rated HSL-44 as "like slightly" or higher. Based on this data, HSL-44 meets the specifications for troop acceptability.

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HSL-44 and the current Army Deet formulation were both tested for compatibility with military materials. These included plastics, adhesives, rubber and elastomers, organic coatings, metals, leather, and textiles. For most materials, the effect of both HSL-44 and the current Army Deet formulation were identical. Four materials were more severely effected by the Army Deet formulation than by HSL-44; surgical tape, methyl methacrylate sheet, polyurethane sheet, and polyurethane varnish.

Primary dermal irritation and dermal sensitization tests were very similar to those cited by the E.P.A. in the "Deet Registration Standard". There was slight transient skin irritation when the material was applied to the skin with an occluded patch. The degree of skin irritation observed on challenge following nine sensitizing treatments and a two week treatment free interval was no greater than on the first exposure. These studies indicate that the prototype formulation is in Toxicity Category IV corresponding to a very low dermal irritation potential. There is no evidence, either in this study or in the literature, that Deet is a sensitizer.

Data cited in the E.P.A. "Deet Registration Standard" clearly show that this material is a potentially severe eye irritant. In most studies a transient corneal opacity is observed, along with prominent chemosis, discharge, and redness. While these effects were observed with the prototype sustained action repellent, there was not a spontaneous recovery within seven days. Washing the material from the eye does reduce but does not eliminate the toxicity. A Deet standard was not included in this test which makes conclusions based on comparison with literature values more tentative. This is particularly significant due to the observation that the formulation with the higher Deet concentration caused less toxicity. These studies should be repeated, and the basis for this toxicity investigated.

A unique product dispensing package is required to help differentiate the new formulation from the current Army repellent. This is crucial since the current Army repellent formulation has poor troop acceptability and thus poor compliance. However, due to the prohibitive expense of producing a prototype plastic bottle, this package was not developed in Phase I. Samples were delivered in white 2 oz. low density polyethylene bottles with a Polytop™ dispensing closure.

The Polytop™ dispensing closure assures ease in dispensing with no possible loss of the cap and is very sturdy. The flip-top spout can be raised and lowered by the thumb of the hand holding the bottle. It is leak proof in the closed position. This closure will be used in the final package. The final package will be a different shape selected by a consumer panel to meet the requirements of the R.F.P. Both the bottle and the closure will be a dark green, brown, or camouflage color, with a silk-screened label.

FOREWORD

In conducting the research described in this report, the investigator(s) adhere to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES

II. INTRODUCTION

A. Objective

The overall objective of this project is to develop a sustained action arthropod repellent formulation with N,N-diethyl-m-toluamide (Deet) as the active ingredient, which remains effective for at least 12 hours and is acceptable to the personnel for which it is intended. In addition, the product must be non-toxic, compatible with materials used by the military and its odor and other physical characteristics must not be so obvious as to indicate position to any possible enemy. At the completion of Phase III the new product will be registered with the Environmental Protection Agency (E.P.A.) and pilot plant manufacturing verified.

BIOTEX, Inc. has developed a sustained action arthropod repellent which meets the requirements of the project. Under normal laboratory conditions the product is efficacious for greater than 12 hours, and is effective for at least 10 hours in extreme environmental conditions. Toxicology studies indicate that the material is safe for use, is compatible with military materials, and is acceptable to users. This report describes the development of this material in Phase I of the overall project.

B. Background

The need to protect troops against arthropod-borne diseases with a minimum expenditure of resources is well recognized. The effectiveness of N,N-diethyl-m-toluamide (Deet) as an arthropod repellent is well established both by the Army and commercial manufacturers. However, pure Deet offers protection for only a few hours at a time. Repeated application is necessary to insure protection during the most critical periods between dusk and dawn, when arthropods are most active. Not only is repeated application a nuisance to soldiers under demanding field conditions, it is also a hazard which may distract them from critical operations and alert the enemy to their position. In general, most products which have a short duration and require repeated application cannot be used during sleep and therefore inherently harbor the seeds of non-compliance. Heavy perspiration, water exposure, abrasion with clothing, and friction against body parts further reduce the effectiveness of Deet.

It is well known that the duration of protection of native Deet is only a few hours. The most conclusive studies of protection time were conducted by Hill, et al., (1979) at LAIR where both a minimum effective dose and protection time were determined for the repellent. The protection time at the minimum effective dose of 26 ug/sq. cm. was found to be 4.8 hours in man. These facts point to the need for a controlled-release arthropod repellent formulation which gives protection for at least 12 hours during the critical period between dusk and dawn when arthropods exhibit their greatest avidity.

A great deal of effort has been devoted to improving the duration of repellency of Deet. Carillo (1972) examined approximately a dozen film forming polysaccharide esters of fatty acids for their capacity to bind Deet. Repellency tests conducted by the USDA showed two of the films to be approximately twice as effective as Deet alone. Khan, Maibach and Skidmore (1975a) evaluated the effect of perfume fixatives (synthetic musks) on protection time of Deet. They found that protection increased from 12 to 88% depending on the musk and the ratio it was mixed with the repellent. In a follow-up study, Khan, et al., (1975b) studied mixtures of Deet combined with vanillin in four different ratios. Vanillin increased the protection time by more than 100%, suggesting that it is feasible to obtain protection against mosquitoes for almost 24 hours. Vanillin, however, has a strong distinctive odor which is easily detected at long distances; much greater than 5 ft.

Khan et al., (1977) attempted to improve water washability and abrasion resistance of Deet by formulating it with Aeroplast dressing (Parke-Davis Co.) which contains copolymers of hydrovinyl chloride-acetate and sebacic acid. Formulation was most effective with triethylene glycol monohexyl ether. Deet in formulation was improved in water washability and resisted abrasion significantly. In four of five tests, Deet with polymer remained effective for approximately 24 hours.

Reifenrath and Rutledge, (1983) studied the properties of 24 Carboset acrylate polymer and 16 silicone polymer formulations with Deet. A selected number of formulations were evaluated for duration of effectiveness against Aedes aegypti mosquitoes in vitro and in animal test systems. Only one formulation appeared to provide greater duration of protection against mosquitoes than unformulated Deet. Water resistance was also improved with the acrylate polymers, and several were significantly more persistent than native Deet.

The Request for Proposal describes additional, albeit unpublished, studies with 24 proprietary microcapsule type formulations. Limited information is provided about the composition and size of the microcapsules. The results of these studies apparently confirm that controlled release formulations can extend the persistence of Deet on the skin.

C. Specific Aims

The specific objectives of the Phase I effort were:

1. Finalize development of the new controlled-release system for Deet and prepare a new batch using radioactive Deet for further testing. Prepare additional microcapsule systems for the controlled-release of Deet.

2. Determine the rate and duration of evaporation of Deet from the new system using the Reifenrath penetration-evaporation cell, and radioisotope measurement.

3. Develop formulation for delivering the controlled release system.

4. Determine the duration of repellency on human volunteers of military age using Aedes Aegypti mosquitoes.

5. Perform sensory evaluation and troop acceptance studies on the repellents.

6. Determine the toxicity of the final formulation which will be used for field studies.

7. Develop the necessary package design and prepare appropriate labels.

III. ACCOMPLISHMENTS

A. Development of Methods for Deet Analysis

1. Gas Chromatography Method

A gas chromatographic method based on the method of Sarmiento and Beroza (1975), was developed for use in both the in vitro test protocol and for determining Deet concentration in controlled release formulations. A Perkin Elmer Sigma 2-B gas chromatograph, equipped with a flame ionization detector, was used with a 45 cm x 2 mm ID stainless steel column packed with 0.1% SP-1000 on 80/100 Carbopack C support (Supelco, Inc., Bellefonte, PA). The column was operated isothermally at 225°C after conditioning. The injection port and detector were both maintained at 250°C. Nitrogen carrier gas flow rate was 40 ml/minute.

N,N-diethyl-m-toluamide, Deet, was diluted with ethanol to make up 0.1%, 0.001%, and 0.0001% w/v solutions. For each analysis, 2 ul of solution was injected into the G.C. Analyses were done in triplicate and averaged. Peak areas were measured with a Sigma 10-B data station and contents calculated using peak normalization.

Three peaks were observed with retention times of 4.0, 6.0, and 9.8 minutes. The 9.8 minute peak area averaged 98.9% of the total area. The minor peaks are either the o or p isomers or contaminants. A sample chromatograph is shown in Figure 1.

A representative standard curve is shown in Figure 2. Response was linear down to a Deet concentration of 5 mg in 2 microliters. Below this level, response and reproducibility were unsatisfactory.

To ascertain that the evaporation/penetration apparatus was operating efficiently, preliminary tests were performed using Deet on hairless mouse skin. The G.C. method was used for these tests. The high concentrations of Deet used assured satisfactory sensitivity of the gas chromatograph. Later tests with lower concentrations did not yield meaningful data due to the lack of sensitivity of the G.C.

Reifenrath and Robinson (1982), in their study of the evaporation/penetration of Deet on human cadaver skin, found an evaporation rate of 1.2 ug/cm^2/hr at 12 hours. When measuring levels using the G.C. method, this is equivalent to a Deet concentration of 5.5×10^{-5}% w/v, which is well below the 2.5×10^{-3}% w/v limit of sensitivity of the equipment. Therefore, more sensitive radiometric methods were used in all in vitro studies.

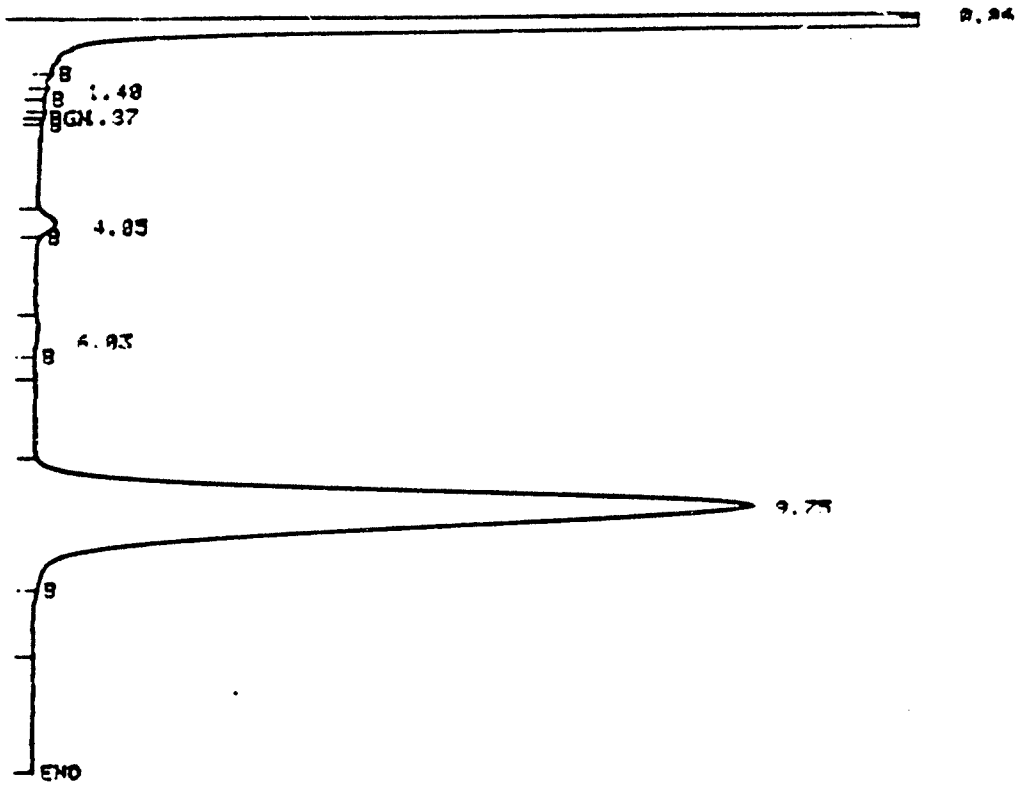
2. Radionuclide Method

Radiolabeled Deet [ring-^{14}C (U)] was prepared as a custom synthesis by New England Nuclear, Boston, MA, at a specific activity of 4.35 mCi/mole. The specific activity was confirmed by comparison to a ^{14}C standard estradiol previously used in this laboratory. The labeled Deet has been shown by New England Nuclear to be 98-99% radiochemically pure by both thin layer chromatography, hexane:ether:acetic acid (70:30:1) on silica gel G, and by gas chroma-

Figure 1

Gas Chromatograph - Deet

2 mm x 45 cm; 0.1% SP-1000 on 80/100 Carbopack C at 225°C, N_2 at 40 ml/min.



PERKIN ELMER PART No. 332 911

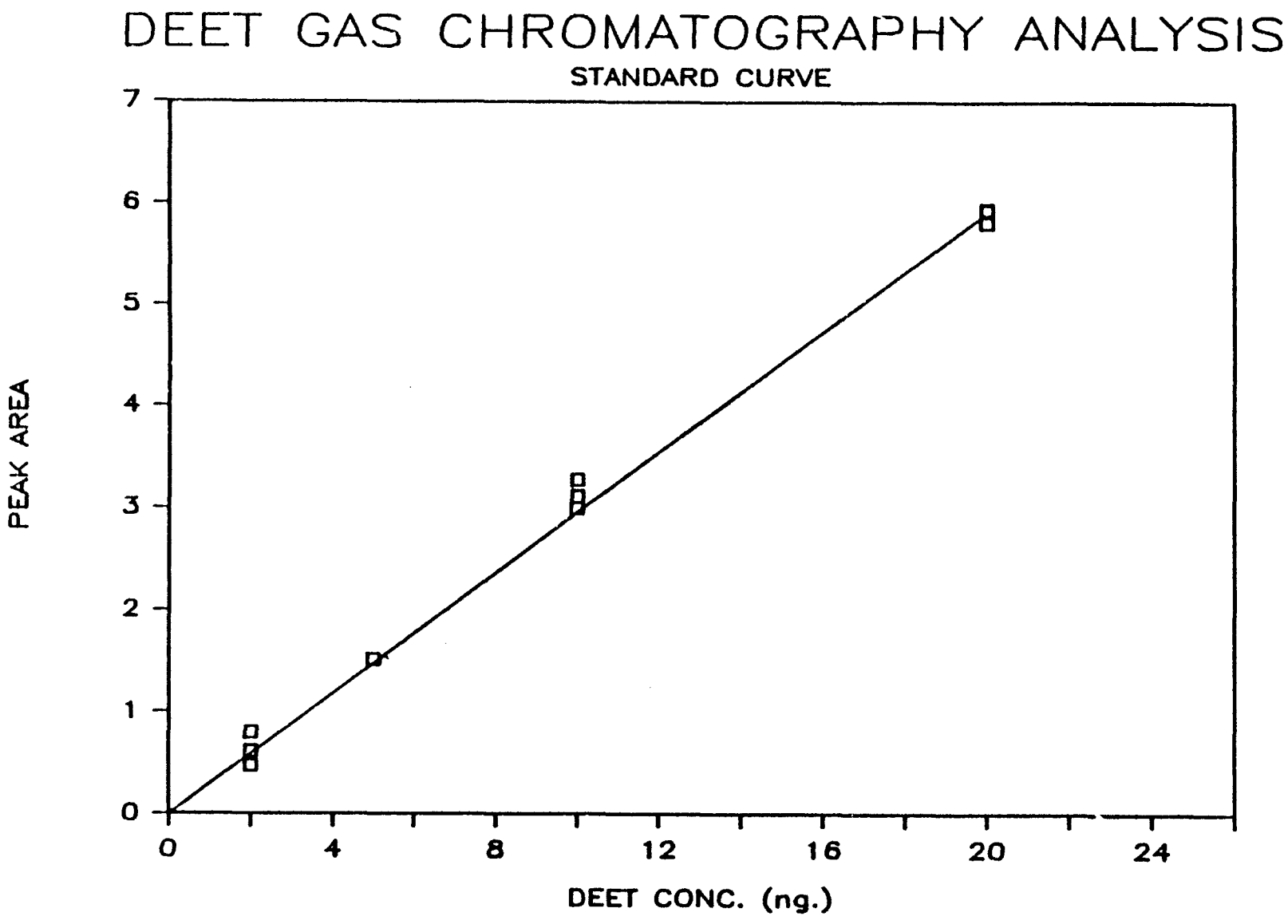
INST 1 METH 1 FILE 13

RUN 2 EVAPORATION TEST 10: 6.5 12/11/84

SENSITIVITIES 50

TIME	AREA	BC	RRT	RF	C	NAME
4.05	0.0403		0.415	1.000	0.7494	-
6.03	0.0148		0.618	1.000	0.2755	-
9.75	5.3305		1.000	1.000	98.9751	DEET

Figure 2


DEET GAS CHROMATOGRAPHY ANALYSIS
STANDARD CURVE
PEAK AREA
0
1
2
3
4
5
6
7
0
4
8
12
16
20
24
DEET CONC. (ng.)

tography. This material was diluted in alcohol and nonradioactive Deet to provide the desired specific activity for each application.

For these studies, ScintiVerse Bio HP scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was selected. This cocktail provides high efficiencies even when there is phase separation, accepts high salt loadings, and is compatible with tissue solubilizers. Radioactivity was measured in a Beckman, Berkeley,, CA, Model 100C liquid scintillation counter.

For skin penetration and evaporation experiments quench was corrected by the external standard channel ratio (ESR) method. Five scintillation vials were prepared with 200 mg of Tenax™ (Alltech, Deerfield, IL) and 64,700 dpm of ^{14}C Deet. A similar set of vials was prepared with 5 ml of Lactated Ringer's solution instead of Tenax™, and all samples counted. Carbon tetrachloride, 3 ul, was added to three samples in each group and the vials recounted. On successive days more carbon tetrachloride was added until the ESR was reduced to approximately 0.02. Vials with no quencher added were also recounted for control. The quench curves, efficiency vs. ESR, obtained are shown in Figure 3. For both Tenax™ and Lactated Ringer's solution the curves are linear. With Tenax™ the equation of the regression line efficiency = 0.0137 (ESR) + 0.949 and r = 0.957, and with lactated ringer's solution efficiency = 0.0276 (ESR) + 0.915 and r = 0.976. In the initial series of experiments the actual efficiencies ranged from 92 to 97%.

After solubilizing tissue samples with 2 ml of Scintigest™ (Fisher, Pittsburgh, PA), the ESR's were too low for accurate quench determination. Therefore a series of studies were conducted to determine quench by internal standardization. Approximately 200 mg of mouse skin was placed in each of several scintillation vials and ^{14}C-Deet (0.245 mg, 0.03 uCi) added to each. An average efficiency of 93.3% was observed.

Due to the unavailability of Scintiverse Bio HP during the course of this study, Biofluor scintillation cocktail (New England Nuclear, Boston, MA) was used as a replacement. This cocktail is similar to Fisher Bio HP. Tenax™ weight in each vapor trap was reduced to 100 mg, and Ringer's Lactate volumetric flow rate was reduced to 2 ml/hr. As a result of these changes, new quench curves were generated.

Tenax™, 100 mg, or Lactated Ringer's, 2 ml, were added to 15 ml cocktail. A known quantity of ^{14}C Deet was added and the samples counted. Following the initial count, increasing amounts of CCl_4 were added to induce quench and the samples recounted. A similar experiment was conducted using samples of digested pig skin. The data is shown in Figures 4 and 5 with efficiencies plotted against ESR. For both Tenax™ and Lactated Ringer's a single curve was obtained for the wide ^{14}C channel. The curve is linear from 74% to 99% efficiencies (r = 0.9761). The equation is efficiency = 0.0395 (ESR) + 0.722. For all experiments the observed efficiency was greater than 96%. Similarly, a single curve (Figure 5) was obtained for ^{14}C in digested pig skin. The equation for the curve is efficiency = 0.0344 (ESR) + 0.732 and r = 0.9259. For all reported experiments the observed efficiency for all measured samples was greater than 88%.

Figure 3

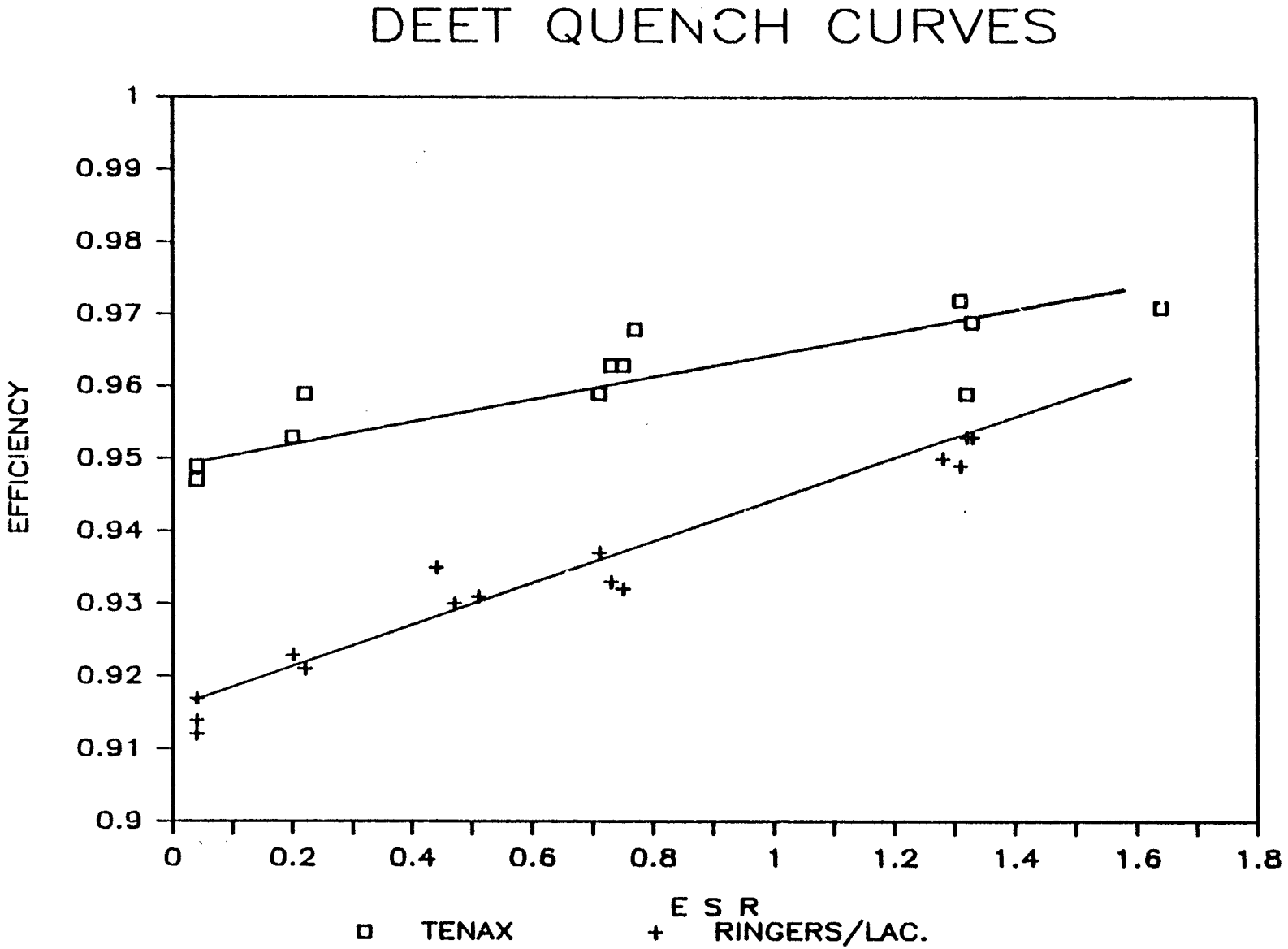

DEET QUENCH CURVES
EFFICIENCY
1
0.99
0.98
0.97
0.96
0.95
0.94
0.93
0.92
0.91
0.9
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
E S R
TENAX
RINGERS/LAC.

Figure 4

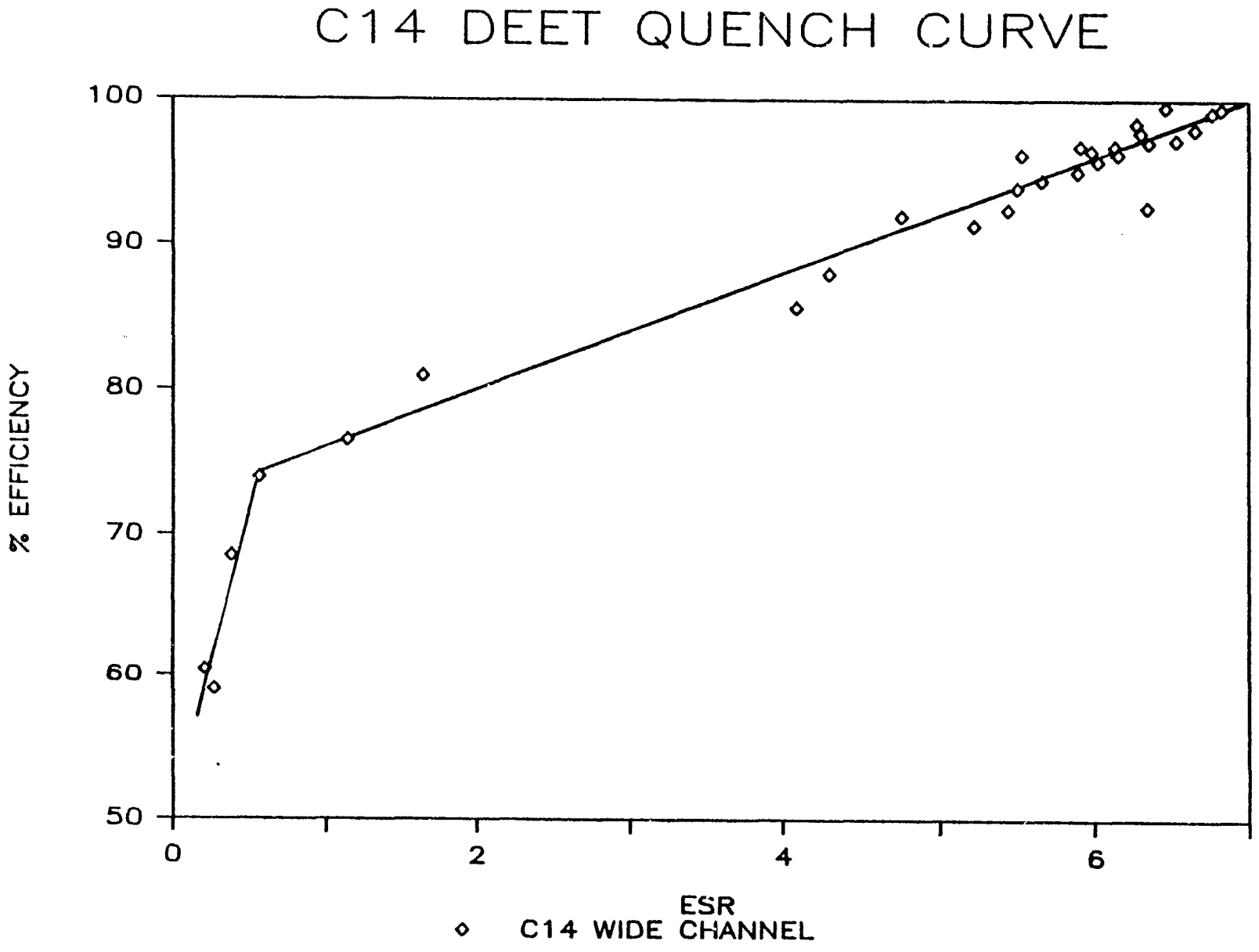
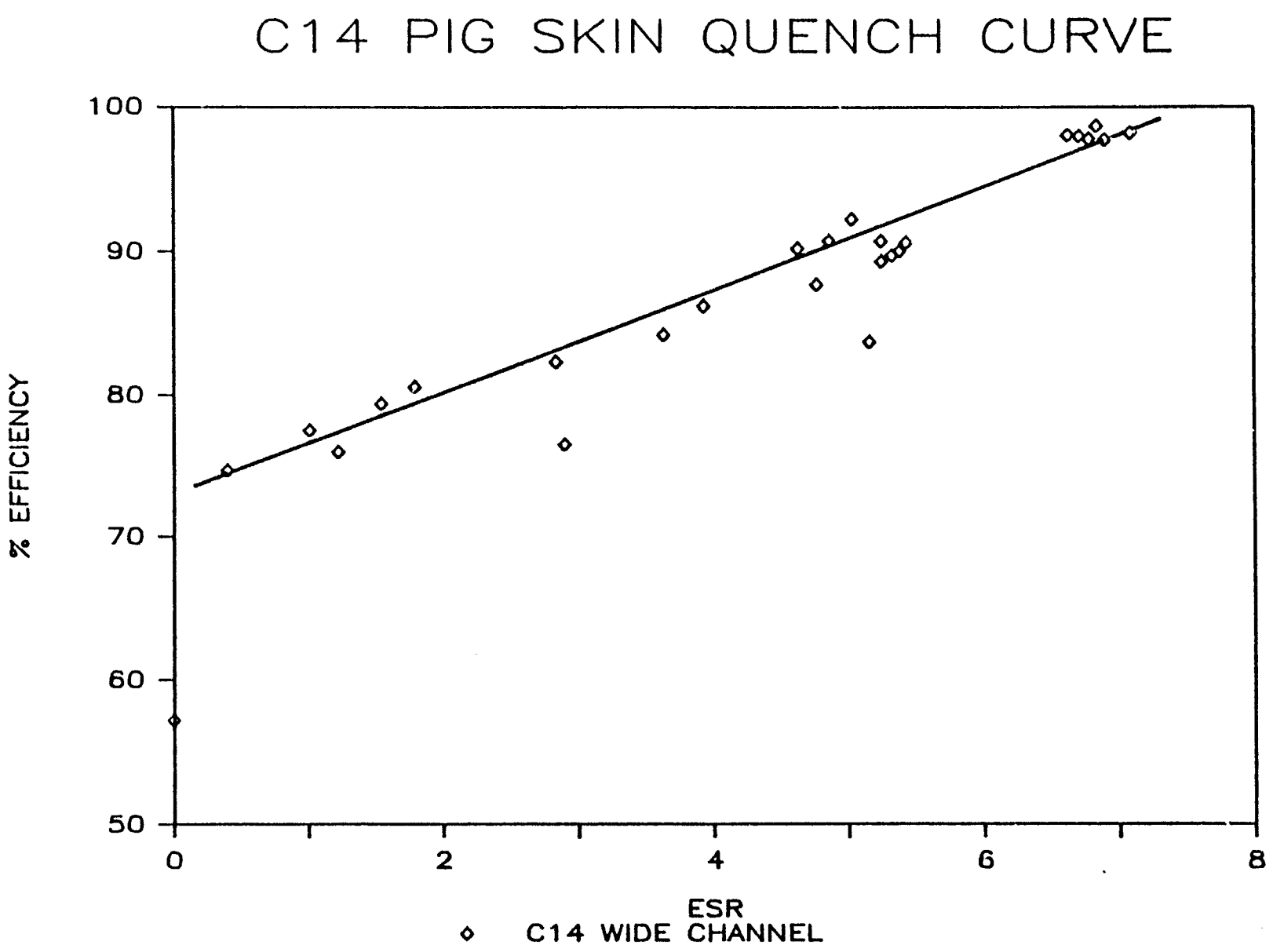

C14 DEET QUENCH CURVE
100
90
80
70
60
50
% EFFICIENCY
0
2
4
6
ESR
C14 WIDE CHANNEL

Figure 5


C14 PIG SKIN QUENCH CURVE
100
90
80
70
60
50
% EFFICIENCY
0
2
4
6
8
ESR
C14 WIDE CHANNEL

B. In Vitro Studies

1. Methodology

Evaporation and penetration rates of native Deet and controlled release formulations were studied using the in vitro system developed by Reifenrath and Robinson (1982) and used at the Lettermen Army Institute of Research (LAIR).

The apparatus used is pictured in Figure 6. A detailed diagram of one evaporation/penetration cell is shown in Figure 7. Freshly excised abdominal skin from the hairless mouse was placed over the lower (penetration) cell, visceral side down, and retained with an o-ring, making sure all air bubbles were removed from under the substrate. The upper (evaporation) cell was clamped on top of the substrate. Ringers lactate was perfused through the lower cell at a rate of 5 ml/hour with a counting vial used to collect the outflow. After 20 minutes, the upper cell was removed to allow application of the desired dose of Deet or controlled release Deet by syringe. The evaporation manifold was then replaced. The lower cell was maintained at 37°C, while the upper cell was maintained at ambient air conditions, 22°C. Air dried by passing over silica gel was drawn into the upper cell, over the surface of the skin, and through a vapor trap packed with 200 mg of absorbant Tenax™ G.C. by a peristaltic pump. Both the vapor trap and counting vial were changed at hourly or bi-hourly intervals. Materials and supplies used in in vitro procedures are listed in Appendix I.

The contents of each vapor trap were placed in counting vials and the traps were rinsed with 15 ml of counting solution which was then added to the vials and the samples counted. Each of the vials used to collect the lower cell outflow were counted after adding 15 ml of counting solution. In each case, the resulting counts per minute were corrected for loss in efficiency due to the presence of either the Tenax™ or Ringer's Lactate to give disintegrations per minute.

If the apparatus was operated with an interval longer than one hour between changes of the vapor trap and lower chamber outflow collector (as in overnight operation), the vapor trap was left intact during the interval and the lower chamber outflow was run into a larger container, with a 5 ml aliquot removed at the end of the interval. Mean evaporation and penetration rates during the interval were then determined.

At the completion of the experiment, the skin sample was carefully removed from the apparatus and digested in 2 ml of Scintigest and 0.2 ml water at 50°C for 2 hours. The efficiency of counting was determined by the internal standard method. The evaporation manifold was rinsed with scintillation cocktail and residual Deet determined.

In order to determine the efficiency of trapping evaporated ^{14}C-Deet on 200 mg of Tenax™, an experiment was conducted. Aliquots of 0.07 or 0.24 ug of ^{14}C-Deet were applied to one end of absorption tubes filled with 200 mg of Tenax™ and dry air passed through the column at 30 ml/minute for one hour to mimic experimental conditions. The study was done in triplicate. For both the high and low applications of Deet, the recovery observed following extraction in Scintiverse Bio HP and counting was 96% ± 2 (S.E.). In the initial series of

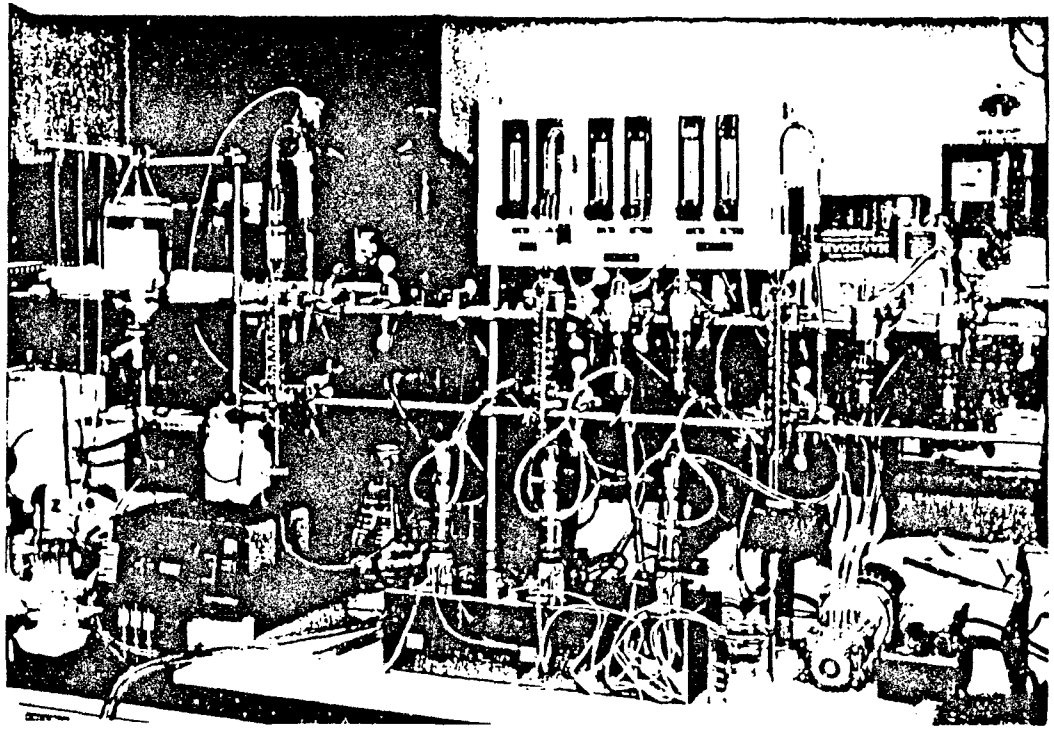
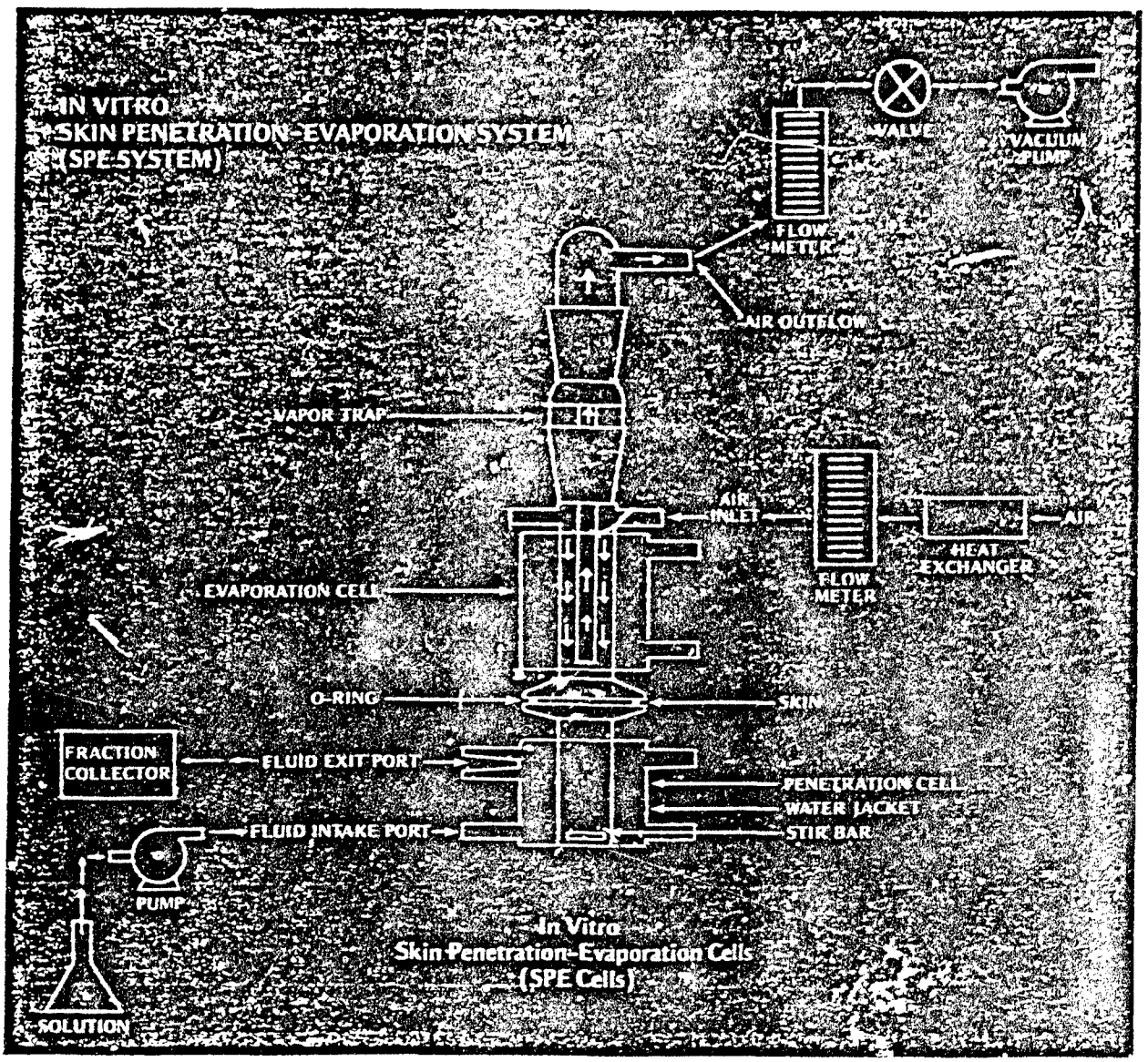


Figure 6 EVAPORATION/PENETRATION APPARATUS

Figure 7 IN VITRO SKIN PENETRATION-EVAPORATION SYSTEM



pilot studies either a secondary Tenax™ trap was included, or the effluent air was bubbled through traps containing scintillation cocktail. In no case was radioactivity found in any secondary trap indicating 100% trapping efficiency.

Due to the high cost of Tenax™ ($5 per gram), a test was performed to determine if the amount of Tenax™ in the vapor trap could be reduced from 200 to 100 mg without any effect on Deet retention. A ^{14}C labeled Deet evaporation/penetration experiment was done on hairless mouse skin using 100 mg Tenax™ vapor traps. A 200 mg secondary trap was placed after the primary trap to absorb any excess Deet which was not absorbed on the primary trap. The secondary trap was left intact for 24 hours while the primary trap was changed hourly. At the conclusion of the experiment the secondary trap was assayed. No radioactivity was detected, indicating that the 100 mg primary trap absorbed all evaporated Deet. One hundred milligrams of Tenax™ were used in each vapor trap in all subsequent tests.

To determine the recovery efficiency of Deet absorbed on 100 mg of Tenax™, ^{14}C labeled Deet was placed on a 100 mg packed Tenax™ column. Dry air was then passed through the column at 30 ml/minute. After one hour, the column contents and rinse were counted to determine how much of the Deet originally placed on the column was recovered. Three levels of Deet on Tenax™ were tested: 2.45 ug, 24.5 ug, and 245 ug, equivalent to 1%, 10%, and 100% of the applied dose in previous tests at 0.3 mg Deet/cm² on hairless mouse skin. The results are listed below:

Column Loading	% of Applied Dose	% Recovery
2.45 ug	1%	108%
24.50 ug	10%	97.3%
245.00 ug	100%	98.9%

Variability in recovery at the lower applied doses can be attributed to error in the application of Deet to the Tenax™. The data shows that essentially all Deet is extracted from the Tenax™.

The high salt loading in Ringers Lactate caused the formation of two phases when mixed with scintillation cocktail. Reasonably high efficiencies were obtained even with two phases. Efficiencies were not stable over a forty-eight hour period though; efficiency increased while the ESR remained constant. Because of this variation, the Ringer's Lactate penetration cell flow was reduced to 2.0 ml per hour. At this concentration of Ringer's in cocktail, the efficiency was stabilized within one hour, permitting more accurate analyses.

2. Revised Methodology

Based on suggestions made by Dr. John Reinert and Dr. William Reifenrath, two major changes in the *in vitro* methodology were instituted. The flow rate of air in the evaporation system was increased to 600 ml/min, and split thickness (1 mm) weanling pig skin was used as the substrate. In addition,

due to supply problems, a new cocktail, Biofluor (New England Nuclear), was used. The liquid scintillation counter also required major repairs. Therefore, new quench correction curves were required.

Skin was obtained from the backs of approximately 25-pound Yorkshire weanling pigs. The skin was shaved with electric clippers and trimmed with a Padgett electric dermatome to a thickness of 1 mm. The skin was mounted in an LGA evaporation/penetration cell with a flow of 600 ml/min of dry air over the skin. The dermal side was perfused with Lactated Ringer's solution at a rate of 2 ml/min. The temperature of the lower cell was maintained at 37°C and that of the evaporation cell at room temperature. For each experiment, labeled ^{14}C Deet or sustained release Deet was applied to the substrate. The evaporation trap containing 100 mg of Tenax™ was changed at 2-hour intervals and after overnight experiments. The Tenax™ was rinsed from the trap with 15 ml of cocktail into a plastic scintillation vial. At the completion of the experiment the total volume of Lactated Ringer's solution was measured and a 2 ml aliquot removed and added to 15 ml of cocktail. The skin was removed and digested at 50°C overnight with 2 ml of Protosol (New England Nuclear) and 200 ul of water in a glass scintillation vial. Biofluor cocktail, 15 ml, was added to the digested samples. All samples were counted in a Beckman LS100C scintillation counter and quench was corrected by the external standard channel ratio (ESR) method. All experiments were conducted in duplicate.

In order to quantitate the ^{14}C Deet evaporated, Deet was extracted from the Tenax™ with scintillation cocktail. Variability in the data from pilot studies indicated that extraction was not instantaneous. Tenax™ samples, 100 mg, were placed in traps and several doses of ^{14}C Deet applied. Air flowed through the traps at 600 ml/min for from 2 to 18 hours. A secondary trap was installed to insure complete recovery. No radioactivity was observed in the secondary traps. At the completion of the experiment the Tenax™ samples were placed in cocktail, mixed, and counted repetitively once per hour for 20 hours. Two representative experiments are shown in Figure 8 for applied doses of 300 and 3000 dpm. Throughout the counting period there was no change in the rate of counting for the standards, which were not absorbed on Tenax™. The initial counts for the absorbed samples were high and then decreased significantly indicating chemiluminescence. At the low dose, recovery was 100% from hours 1 through 20. However, at higher dose counting rates increased after the first hour, and it was apparent that 7 hours were required for extraction. Similar results were obtained for doses up to 80,000 dpm. Mean recovery of Deet from Tenax™ was 91.9% (± 2.7% s.e.m) in 4 experiments. Therefore all samples were extracted at least 7 hours before counting.

3. Skin Evaporation/Penetration Studies

a. Experiments with the Initial Method Using Hairless Mouse Skin

1) Native Deet

The first use of the evaporation/penetration apparatus was to duplicate the work of Reifenrath and Robinson with modifications. More easily obtainable hairless mouse skin was used as the substrate instead of human cadaver skin. The volumetric flow rate of air over the skin surface was 30 cc/minute for these initial tests. Unless otherwise noted, all applied doses of Deet are approximately 300 ug/cm^2.

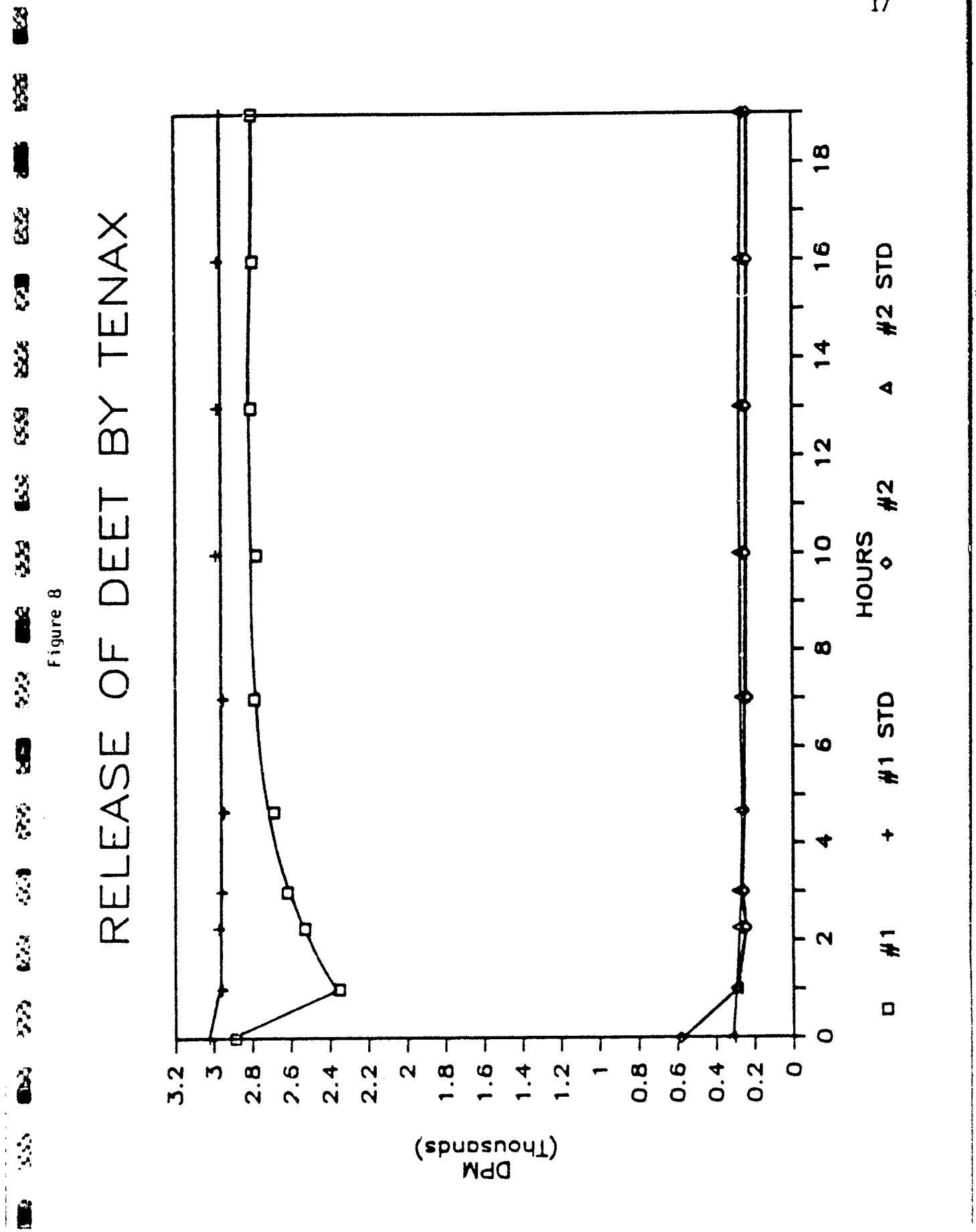

RELEASE OF DEET BY TENAX
DPM
(Thousands)
3.2
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
HOURS
□ #1
+ #1 STD
◇ #2
△ #2 STD

Figure 8

The mean rates of evaporation and the cumulative dose evaporated over the twenty-four hour test period are shown in Figures 9 and 10, respectively. The data of Reifenrath and Robinson are shown for comparison. The initial rate of evaporation was higher for mouse skin; 9.1 + 0.05 (S.E.) ug/cm^2/hr, than for human cadaver skin; approximately 5.8 ug/cm^2/hr. The evaporation off the mouse skin substrate decreased rapidly and ceased after twelve hours. Skin penetration (Figure 11) was very rapid through mouse skin as compared to penetration through human cadaver skin. Data for this experiment is summarized in Table 1.

2) Silica Gel Characteristics

Three silica gels were evaluated in the initial phases to determine which gel would provide the best release profile. The silicas have the characteristics listed in Table 2.

Determination of the Deet absorption capability of the three types of silica gels was done using the ASTM. D 281-31 test method. A small sample of silica (approximately 1 gm) was weighed and placed on a glass plate. Deet (Lot #020684, obtained from Alfa Chemical Corp., Danvers, MA) was added, dropwise, from a burette and thoroughly incorporated into the silica with a stainless steel spatula. The loading capacity of the silica was reached when enough Deet was added to form a stiff, putty-like paste which did not break when cut with the spatula. The volume of Deet required was recorded. Using the specific gravity of Deet, the number of grams of Deet required to fully "wet" the silica was determined. The results obtained are shown in Table 3.

These results indicate that silicas B and C, because of their higher internal pore volume, were capable of "holding" more Deet.

3) Deet/Silica Sustained Release Matrices

The first Deet/silica matrices tested on the Reifenrath apparatus were 1:1 Deet/silica weight ratio blends prepared in the following manner: One ml of 2.45% w/v Deet in ethanol solution (specific activity of 1.19 x 10^{-4} uCi/ug Deet) was added to 24.5 mg of silica and mixed well. Three preparations were made, each containing one of the three silica gels.

Ten microliters of each Deet/silica/ethanol suspension were pipetted onto duplicate or triplicate hairless mouse skin substrate to provide a dose of 0.3 mg/cm^2 of Deet (2.92×10^{-2}uCi). The ethanol evaporated rapidly, leaving a powdery film on the surface of each substrate.

The silica loading at these levels was approximately one-third of the maximum determined above.

For these tests, the air flow rate was 30 cc/min over the skin surface.

The mean evaporation rate profiles are presented in Figure 12. Silica gel A, (n=2), had an initial evaporation rate of 11 ug/cm^2/hr. This rate decreased rapidly to 5.7 ug/cm^2/hr at 5.5 hours. During subsequent intervals the evaporation rate decreased more slowly. The decrease in Deet evaporation rate from silica gels B and C, (n=3 each), was nearly linear throughout the observation period, although the cumulative amount of Deet evaporation (Figure 13) does

Figure 9

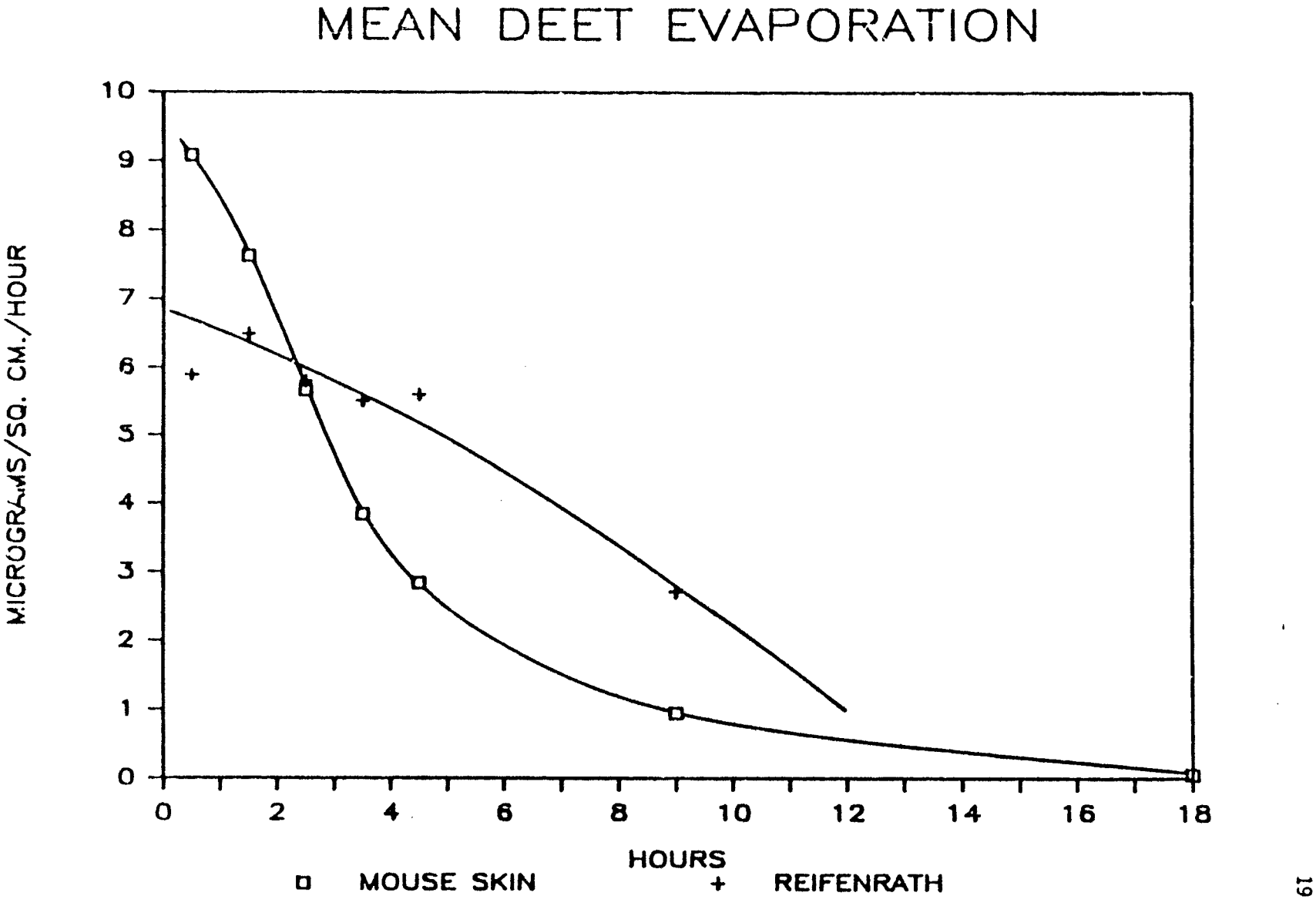

MEAN DEET EVAPORATION
MICROGRAMS/SQ. CM./HOUR
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
HOURS
MOUSE SKIN
REIFENRATH

Figure 10

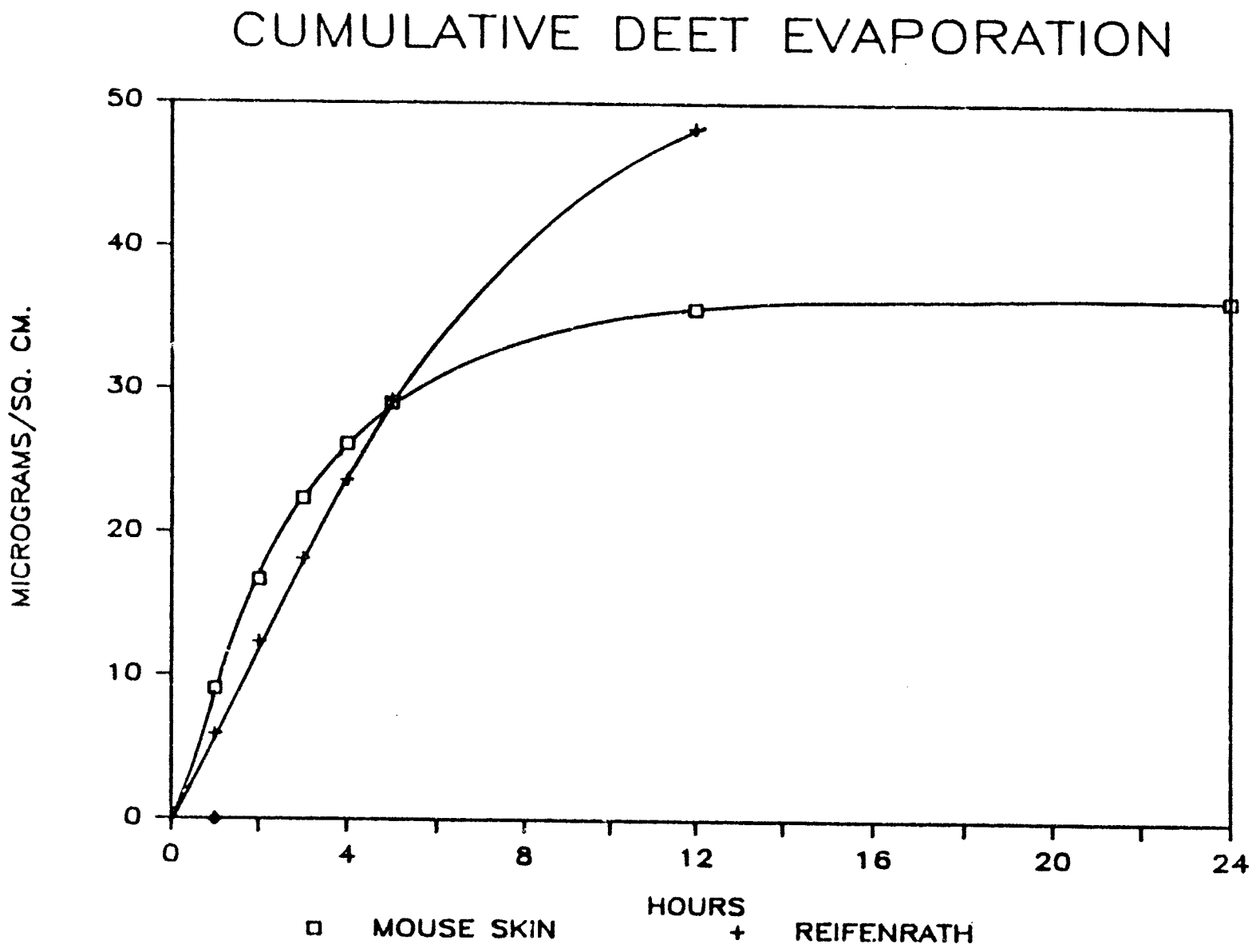

CUMULATIVE DEET EVAPORATION
MICROGRAMS/SQ. CM.
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS
MOUSE SKIN
REIFENRATH

Figure 11

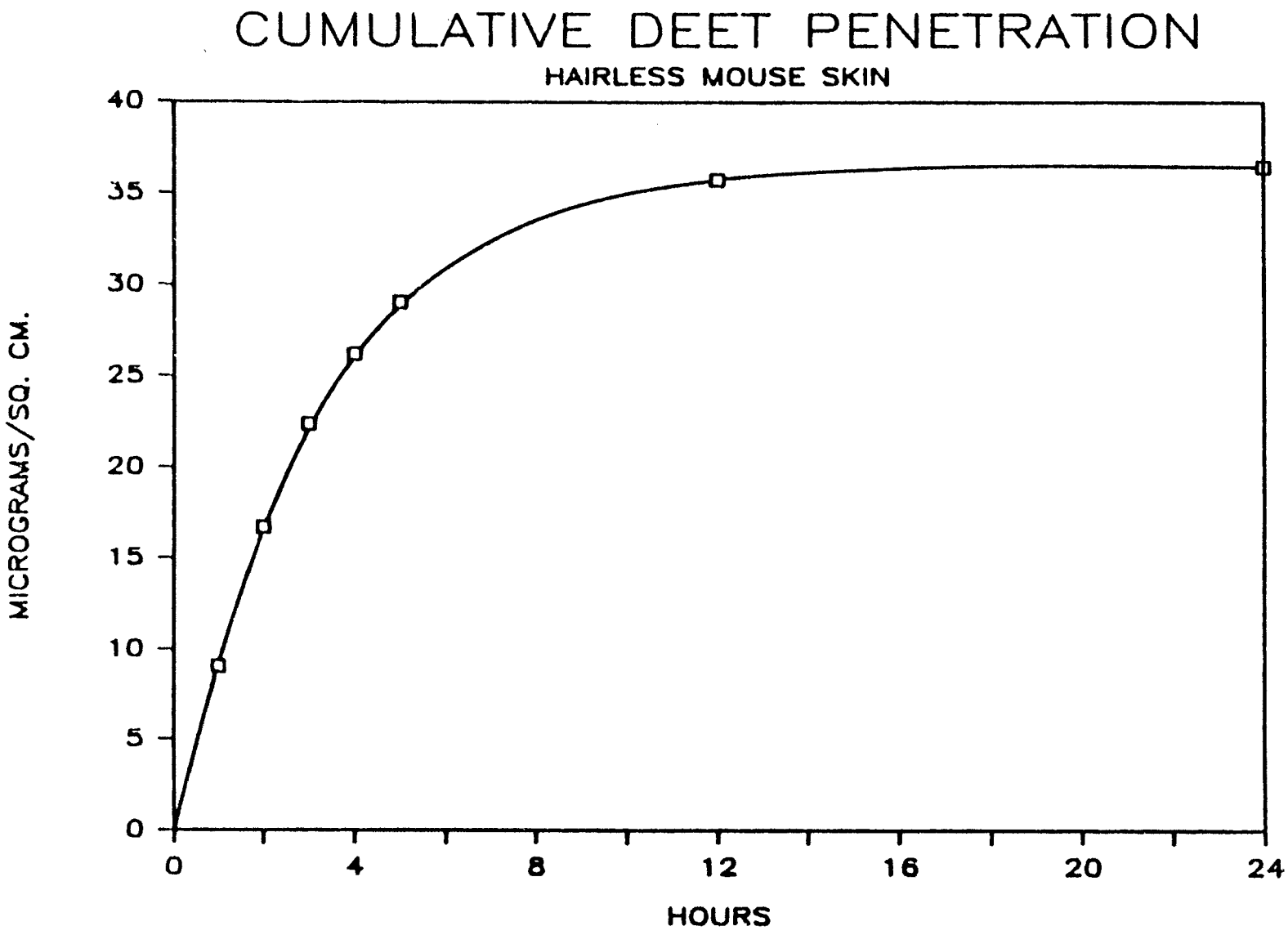

CUMULATIVE DEET PENETRATION
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ. CM.
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
HOURS

Table 1

DISPOSITION OF RADIOACTIVITY 24 HOURS AFTER APPLICATION OF ^{14}C DEET ON FRESHLY EXCISED HAIRLESS MOUSE SKIN (0.30 mg/cm^2)

Sample	Evaporation	Percutaneous Penetration	Skin Digestion	Cell Rinse	Total
Neat Deet (1)	15.5	70.9	4.2	-	90.6
Neat Deet (2)	14.8	75.2	2.6	-	92.6
Neat Deet (3)	8.2	57.5	8.7	6.2	74.6
Mean ± S.D.	12.8 ± 4	67.9 ± 9.2	5.2 ± 3.1		85.9± 9.9

TABLE 2

SILICA GEL CHARACTERISTICS

Type	Average (Particle Size um)	Pore Volume %	Average Pore Diameter Å
Silica A	3	75	150
Silica B	2	85	200
Silica C	15	85	200

Table 3

SILICA GEL LOADING

Type	Deet Loading Capacity
Silica A	2.93 g/g
Silica B	3.28 g/g
Silica C	3.15 g/g

Figure 12

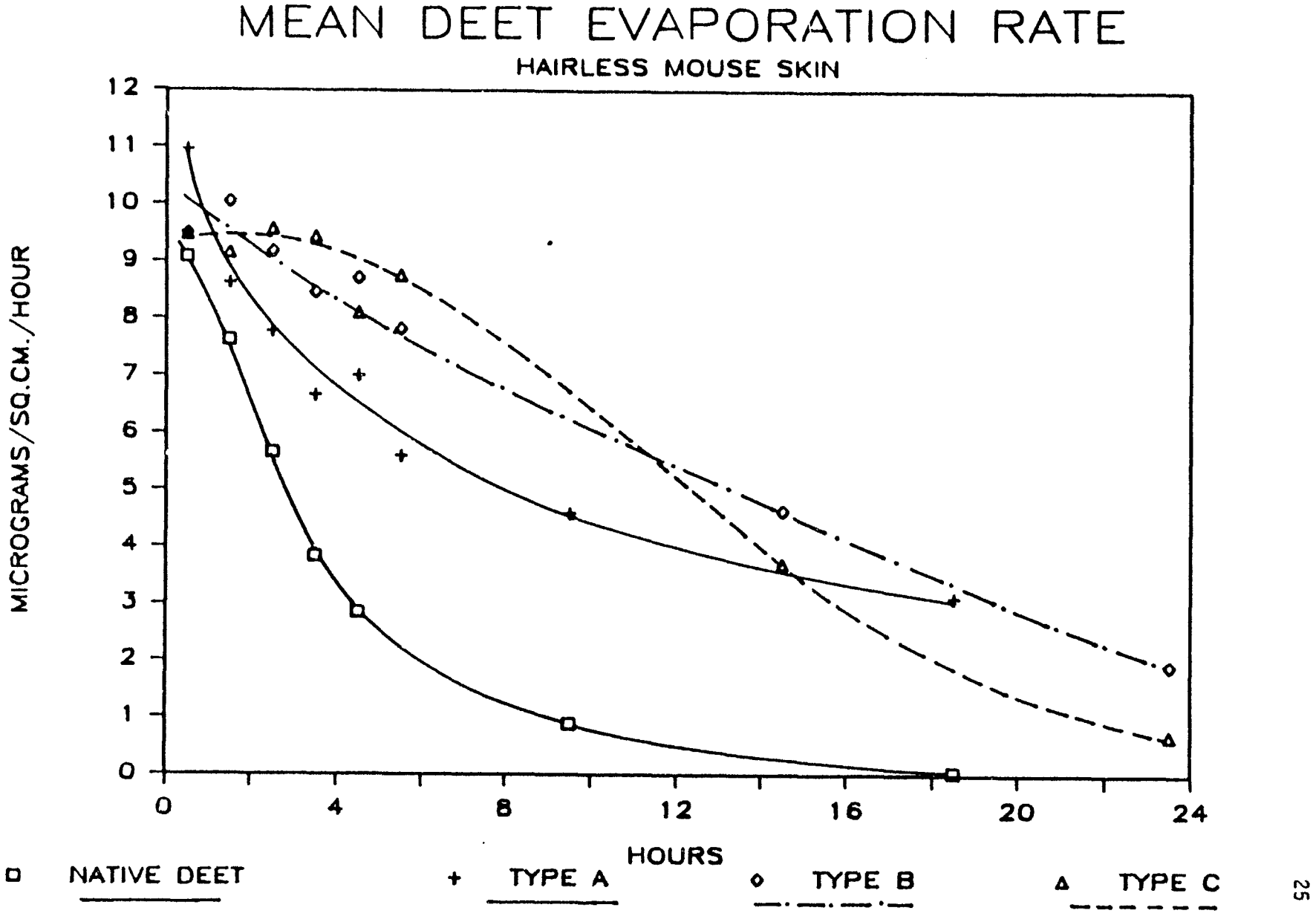
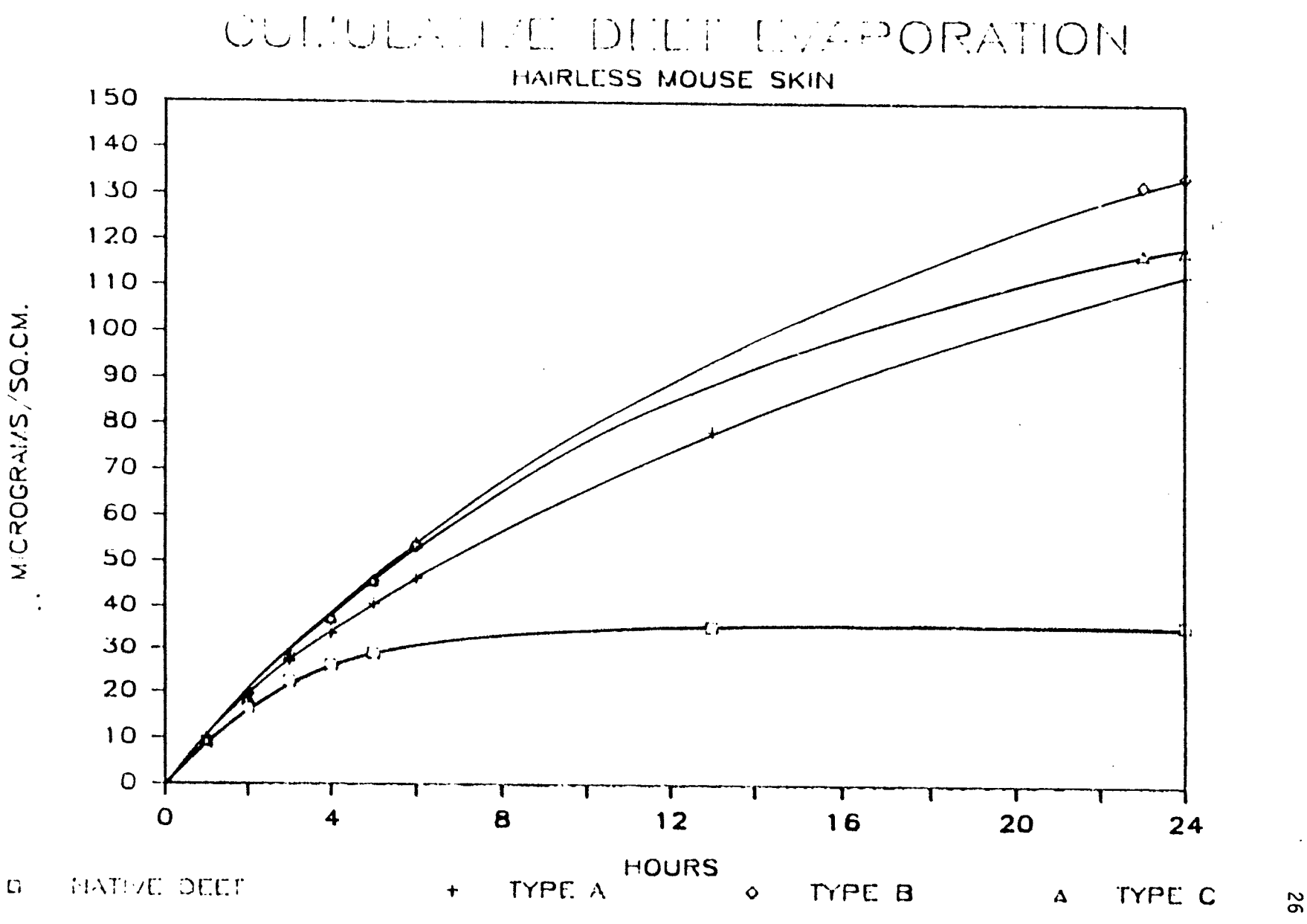

MEAN DEET EVAPORATION RATE
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ.CM./HOUR
0
1
2
3
4
5
6
7
8
9
10
11
12
0
4
8
12
16
20
24
HOURS
NATIVE DEET
TYPE A
TYPE B
TYPE C

Figure 13


CUMULATIVE DEET EVAPORATION
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ.CM.
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS
NATIVE DEET
TYPE A
TYPE B
TYPE C

show a progressive decrease. For silica gels B and C the initial rates were both 9.5 ug/cm²/hr, and at 18 hours the values were 4.2 and 2.5 ug/cm² /hr, respectively. Each of these rates was significantly greater than the minimum effective rate of evaporation, 1.3 ug/cm²/hr, reported by Reifenrath and Robinson (1982).

The mean rate of Deet skin penetration, shown in Figure 14 increased rapidly during the first 3 hours to a maximum of 14 ug/cm²/hr for silica gel Type A and 11.0 and 10.5 ug/cm²/hr for types B and C, respectively. Skin penetration then rapidly decreased and by 18 hours was less than 4 ug/cm²/hr. The cumulative amount of Deet transdermal penetration (Figure 15) was greatest, 131.9 ± 2.4 ug/cm², for silica gel Type A compared to types B and C at 122.1 ± 13.4 and 115.0 ± 10.8 ug/cm², respectively.

Comparison of these results to those obtained with native Deet dissolved in ethanol at the same dose (Figure 15) demonstrates that silica gel absorption does not significantly alter the initial evaporation rate. The period during which the evaporation rate exceeds the minimal effective evaporation rate was extended from approximately 8 hours to more than 18 hours. This prolonged period of evaporation results in approximately a three-fold increase in total Deet evaporated.

In these studies recovery of administered radioactivity ranged from 82.4% to 97.8% (Table 4). The three silica gels had very similar recovery patterns. Compared to Deet in alcohol, Deet absorption on silica gel significantly increased the percent of drug evaporation, decreased penetration and increased the percent remaining on the skin.

4) Deet/Silica Type B Sustained Release Matrices with Polymers

Silica gel Type B was selected for further development, including encapsulation, since it exhibited the most linear evaporation response, the greatest cumulative evaporation, and less penetration than silica gel type A.

Further testing of Deet/silica mixtures was done using hairless mouse skin and human cadaver skin as substrates.

Human skin was obtained at autopsy from Beth Israel Hospital in Boston, MA. A dermatome was not available so the skin was reduced to 2 mm thickness manually. Skin samples were wrapped with gauze, packed in Lactated Ringer's solution and stored at -80°C.

Native Deet, 1:1 Deet/silica mixture, Deet/silica poly-L(-)lactide microcapsules (5% poly-L(-)lactide coating) and siliconized Deet/silica (1% siliconizing fluid) were tested in the Reifenrath apparatus using human cadaver skin as substrate. Air flow rate was 30 ml/min. Due to the inconsistencies in skin thickness caused by manual dermatoming, the results of these tests were inconclusive.

These Deet formulations were also tested on hairless mouse skin at an air flow rate of 30 ml/min. The 50% Deet/silica, Deet/silica poly-L(-)lactide microcapsules, and the siliconized Deet/silica all provided extended evaporation rates above 1.3 ug/cm²/hr for periods in excess of 14 hours. The results are shown in Figure 16.

Figure 14

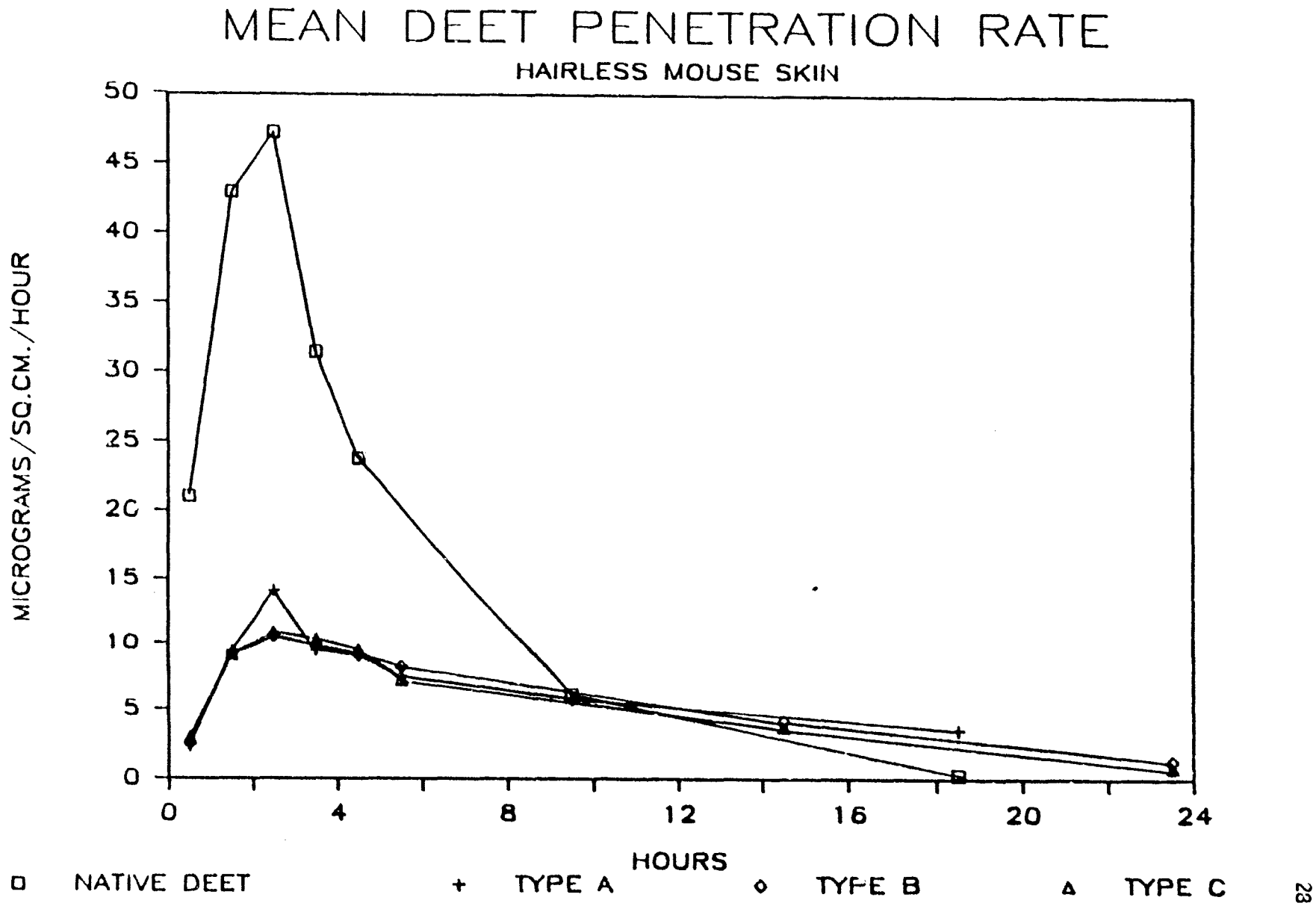

MEAN DEET PENETRATION RATE
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ.CM./HOUR
50
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
HOURS
NATIVE DEET
TYPE A
TYPE B
TYPE C

Figure 15

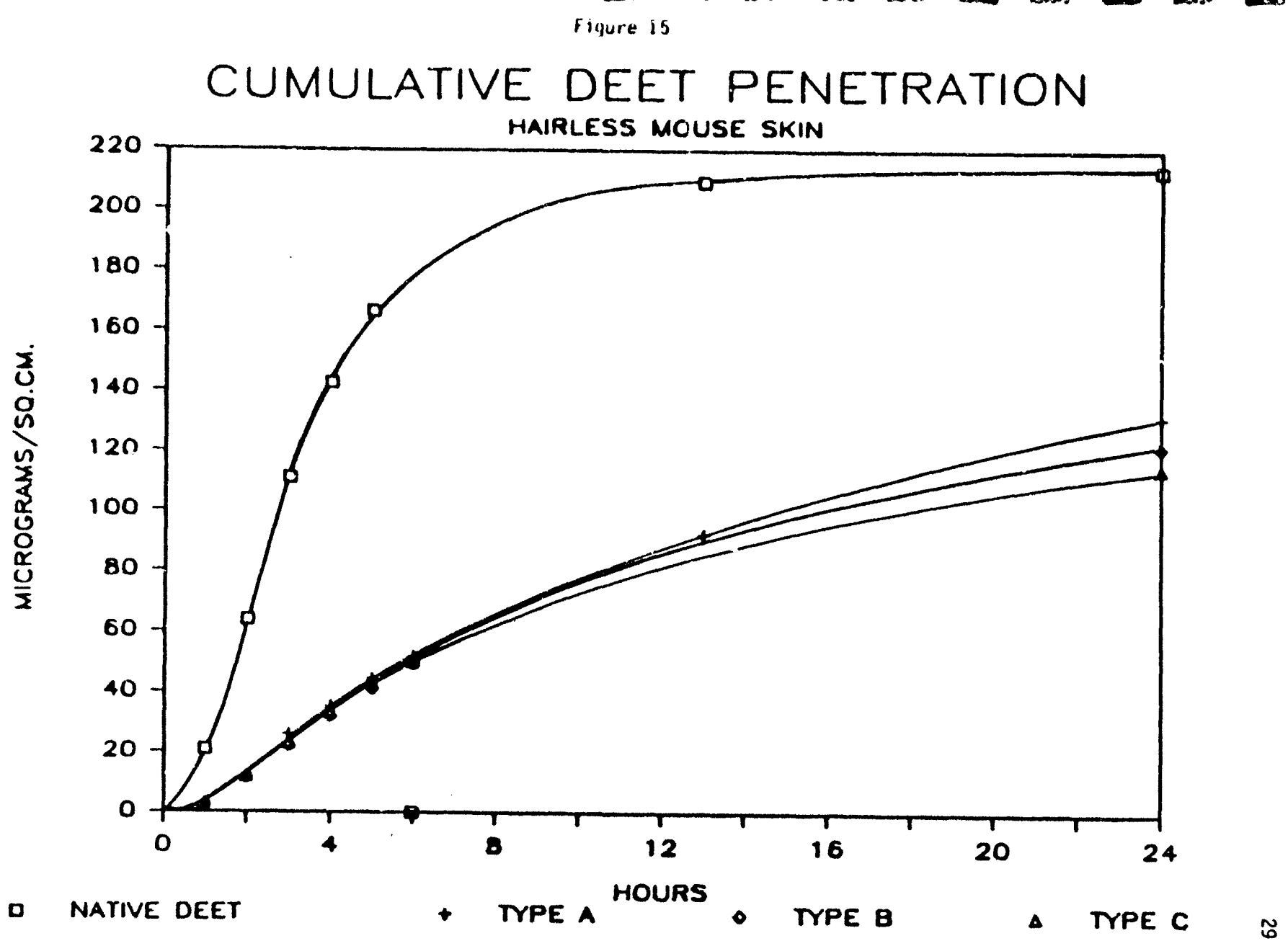

CUMULATIVE DEET PENETRATION
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ.CM.
220
200
180
160
140
120
100
80
60
40
20
0
0
4
8
12
16
20
24
HOURS
NATIVE DEET
TYPE A
TYPE B
TYPE C

Table 4

DISPOSITION OF RADIOACTIVITY 24 HOURS AFTER APPLICATION OF ^{14}C-DEET ON FRESHLY EXCISED HAIRLESS MOUSE SKIN (0.30 mg/cm^2)*

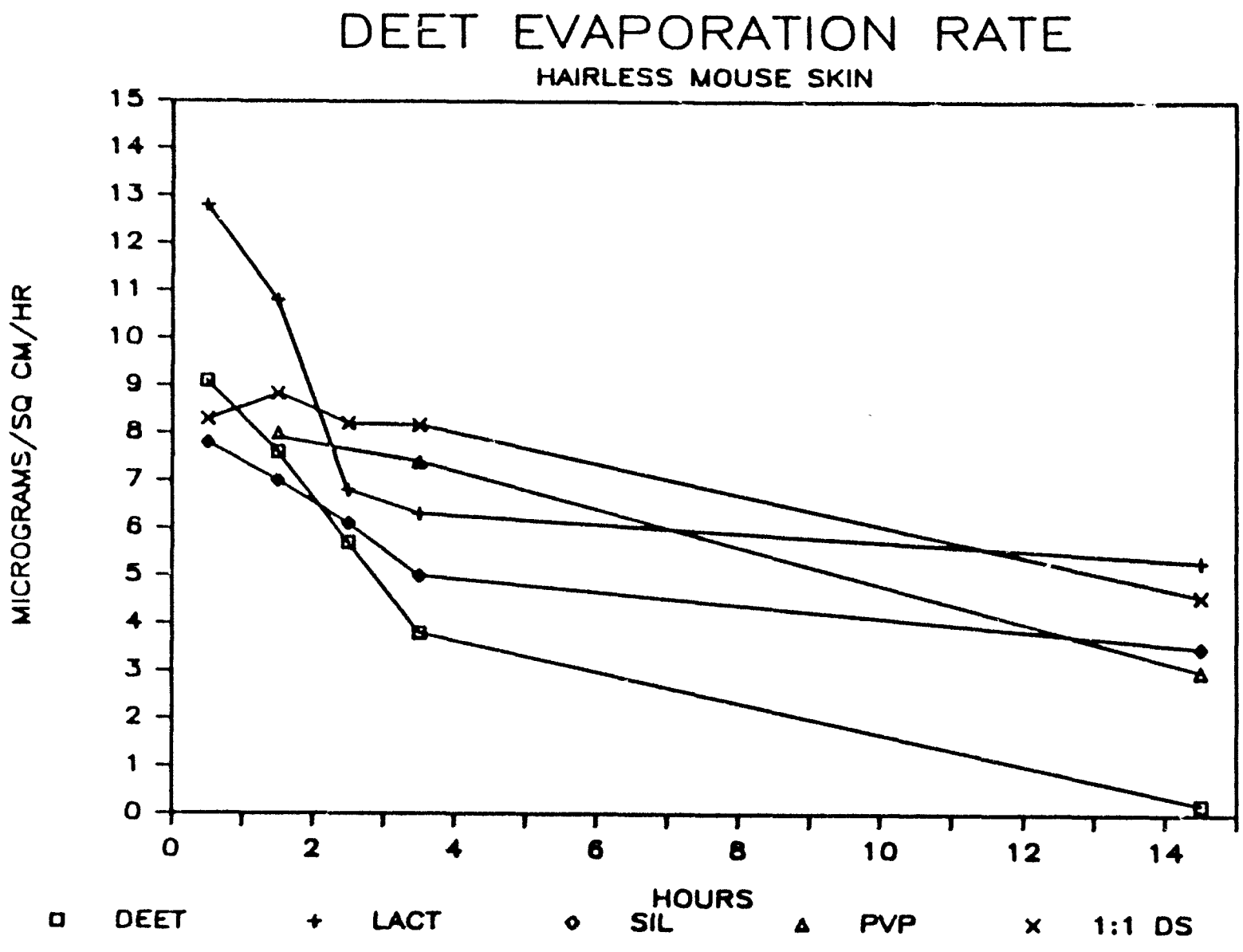
Sample	Evaporation	Percutaneous Penetration	Skin	Cell Rinse	Total
Neet Deet	12.8 ± 4	67.9 ± 9.2	5.2 ± 3.1	(6.2**)	85.9 ± 9.9
Deet/Silica Gel A	36.3 ± 1.9	42.2 ± 1.6	17.5 ± 1.0	1.9 ± 1.6	97.8 ± 2.8
Deet/Silica Gel B	43.0 ± 4.6	39.1 ± 9.1	11.5 ± 5.4	1.5 ± 1.5	95.2 ± 11.3
Deet/Silica Gel C	37.9 ± 9.5	36.8 ± 7.3	9.1 ± 2.6	0.6 ± .04	84.5 ± 11.8
Neet Deet***	16.7 ± 3.7	6.6 ± 2.2	51.9 ± 9.1	----	77.8 ± 2.6

* Data are presented as means ± standard deviation of the percent of the applied dose (245 µg) recovered. N=3 for each experiment except silica gel A where N=2.

** Only one cell was used and rinsed in this experiment.

*** For comparison, the data of Reifenrath and Robinson (1982) for recoveries at 12 hours is included.

Figure 16


DEET EVAPORATION RATE
HAIRLESS MOUSE SKIN
MICROGRAMS/SQ CM/HR
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
HOURS
DEET
LACT
SIL
PVP
1:1 DS

5) Deet/Silica Type B Sustained Release Weight Ratios

To determine more completely the effect of silica gel, Type B, loading on evaporation/penetration characteristics, more tests were done using a variety of loading levels. Using hairless mouse skin as substrate, air flow was maintained at 30 ml/min. The weight ratios shown in Table 5 were tested.

The 50% (by weight) silica provided the highest evaporation rate over the longest period of time. As the loading levels dropped, the evaporation profiles more closely resembled native Deet. As the loading levels increased, the evaporation profiles were more linear at a higher level. Results are shown in Figure 17.

b. Experiments with the Revised Method Using Weanling Pig Skin

1) Modifications

To more closely simulate the in vivo action of Deet on human skin, several changes were made to the in vitro test equipment and protocol. These changes were made on the recommendation of the project officer, Colonel J. Reinert during a site visit on February 6, 1985. The changes include the following:

1. Using weanling pig skin to replace hairless mouse skin;
2. Increasing the air flow rate from 30 ml/min to 600 ml/min; and
3. Raising the minimum evaporation rate from 1.3 ug/cm^2/hr to 5.0 ug/cm^2 hr.

Three new additional Reifenrath cells were purchased to bring the total number of cells to six. This allowed greater flexibility and increased capacity. Another pump and six larger volume flow meters were purchased to permit the recommended 600 ml/min of evaporation air to flow to each of the six cells. The skin surface area in each test cell is 0.785 square centimeters. The equivalent velocity of air over the skin surface was therefore increased from 6.3×10^{-1} m/s to 1.27×10^{-1} m/s. This higher value more closely corresponds to actual in vivo conditions, according to Reifenrath et al. (1984). A new minimum effective evaporation rate of 5.0 ug/cm^2/hr was also reported by Dr. Reifenrath at this point during the study.

Weanling Yorkshire pig skins were obtained from Tufts Veterinary School in North Grafton, MA to replace the hairless mouse skins used in previous evaporation/penetration experiments. Reifenrath et al. (1984) had shown that weanling pig skin closely resembles human cadaver skin in its penetration/evaporation characteristics (Hawkins and Reifenrath, 1984).

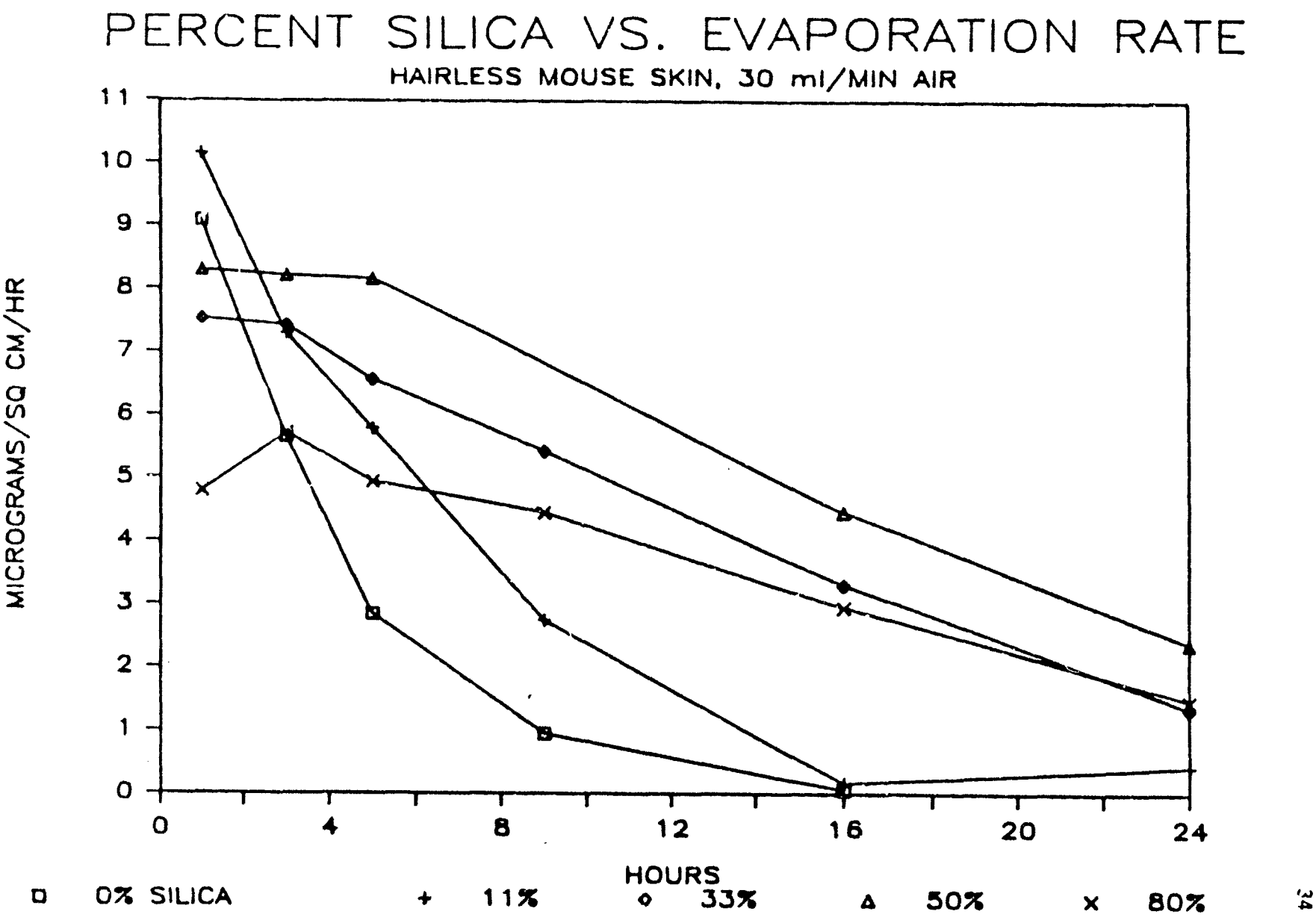
The pigs were sacrificed by lethal injection of barbiturate. Two 15" square pieces were removed from the back and dermatomed to a final thickness of 0.9 to 1.0mm. Pieces were wrapped in gauze, packed in Lactated Ringer's solution and stored at -78°C over dry ice. The maximum length of storage before use was two weeks.

Table 5

SILICA GEL LOADING TESTS

Weight % Silica
0
11
33
50
80

Figure 17


PERCENT SILICA VS. EVAPORATION RATE
HAIRLESS MOUSE SKIN, 30 ml/MIN AIR
MICROGRAMS/SQ CM/HR
11
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
HOURS
0% SILICA
11%
33%
50%
80%

The silica gel loading tests were repeated at a flow rate of 600 ml/min as recommended by Reifenrath, et al. (1984). Hairless mouse skin was used as the substrate. The increased flow caused over half of the mouse skin substrates to rupture.

The first samples of weanling pig skin obtained from Tufts Veterinary School for use as substrate were dermatomed by hand to approximately 1 mm thickness. Native Deet and two Deet/silica mixtures were tested. Because of variations in the thickness of the substrate, the results were inconclusive.

2) Deet/Silica Type B Sustained Release Weight Ratios I

A second set of pig skins was taken to Shriners Burn Institute in Boston, MA, where they were reduced in thickness using a Padgett electric dermatome.

Tested on this pig skin in the Reifenrath apparatus were samples of native Deet, 50% and 33% weight ratio silica Type B/Deet mixtures. The first sample, which was dermatomed to 0.25 mm, resulted in greatly increased penetration of Deet. A third pig was sacrificed, the skin removed and dermatomed to a thickness of 0.9mm. Thickness was verified with an Ames Micrometer.

With this skin as substrate and applications of native Deet, 50% Deet/50% silica gel Type B, and 80% Deet/20% silica gel Type B at the reduced air flow rate of 30 ml/min, both the 50% and 20% silica/Deet mixtures provided extended evaporation above the minimum effective evaporation rates of 5.0 ug/cm^2 /hr. The evaporation profiles are shown in Figure 18.

3) Comparison of Initial and Revised Method Data

Increasing the evaporation cell air flow rate had a significant effect. The evaporation rate of native Deet from weanling pig skin is compared at 30 and 600 ml/min in Figure 19. At 30 ml/min the evaporation rate was stable for ten hours and then slowly decreased. The rate fell below the initial critical effective level described by Reifenrath and Robinson (1982) of 1.3 ug/cm^2/hr at approximately 17 hours. At the higher flow rate, Deet evaporation followed first order, rather than zero order kinetics, and the rate fell below the critical level for this flow rate, 5 ug/cm^2/hr, (Reifenrath, Personal Communication), between five and six hours.

Similar results are shown for sustained release Deet in Figure 20. At a flow rate of 30 ml/min, the Deet absorbed on silica gel evaporated at a rate greater than the critical level for at least 23 hours. When the flow rate was increased to 600 ml/min, the duration of effective evaporation rate was less than seven hours.

The reproducibility of the method is shown in Figure 21 where duplicate determinations of neet Deet evaporation profiles observed during a two-month period are presented. The mean initial evaporation rate is 92.05 ug/cm^2/hr (+ 2.7 ug/cm^2 /hr s.e.m.) and the duration of the effective evaporation rate is five hours.

Qualitatively, the results are similar to those of Reifenrath's group (Personal Communication). Increasing the air flow rate alters the observed kinetics of the evaporation processes and decreases the duration. Quantita-

Figure 18

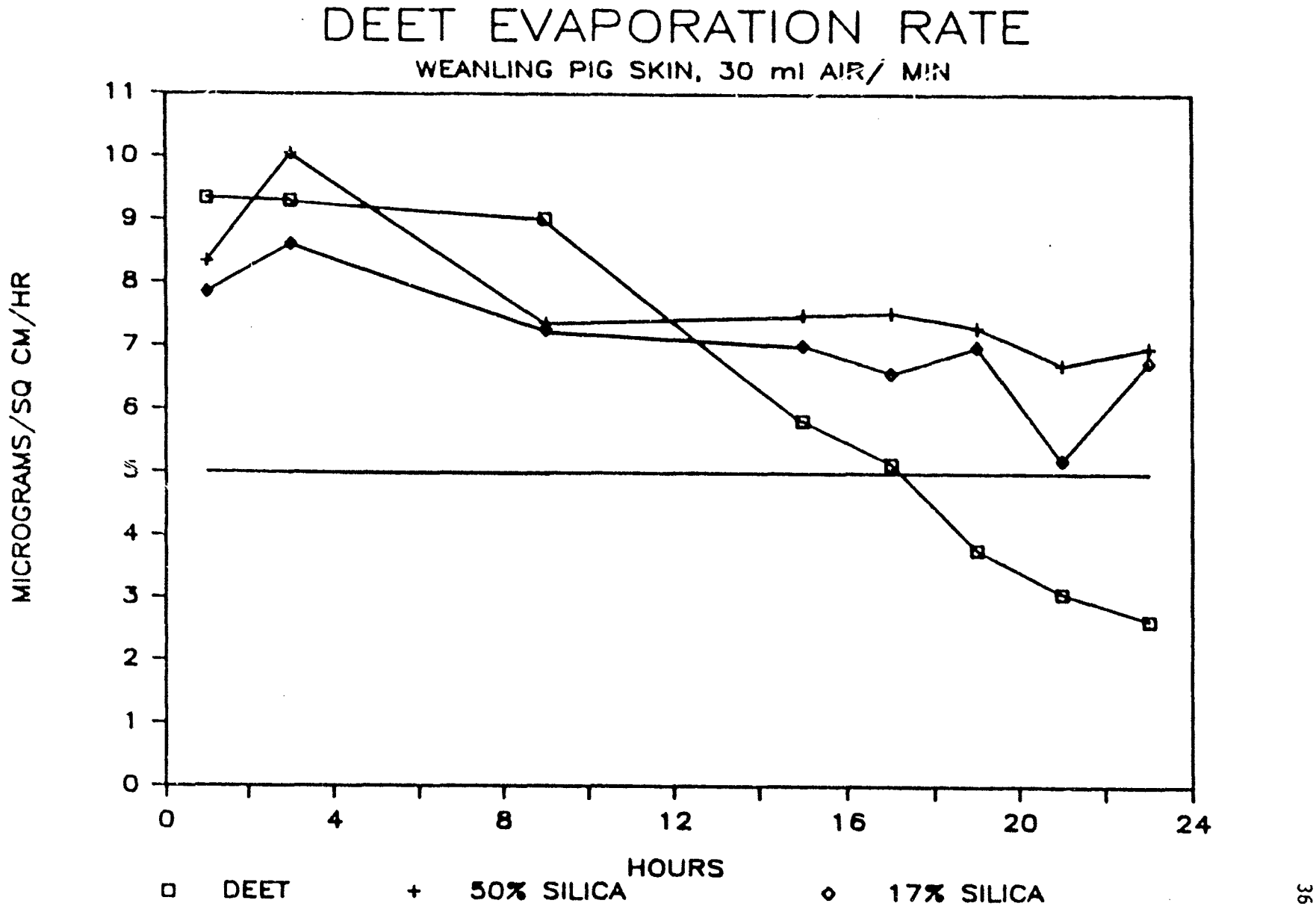

DEET EVAPORATION RATE
WEANLING PIG SKIN, 30 ml AIR/ MIN
MICROGRAMS/SQ CM/HR
11
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
HOURS
DEET
50% SILICA
17% SILICA

Figure 19

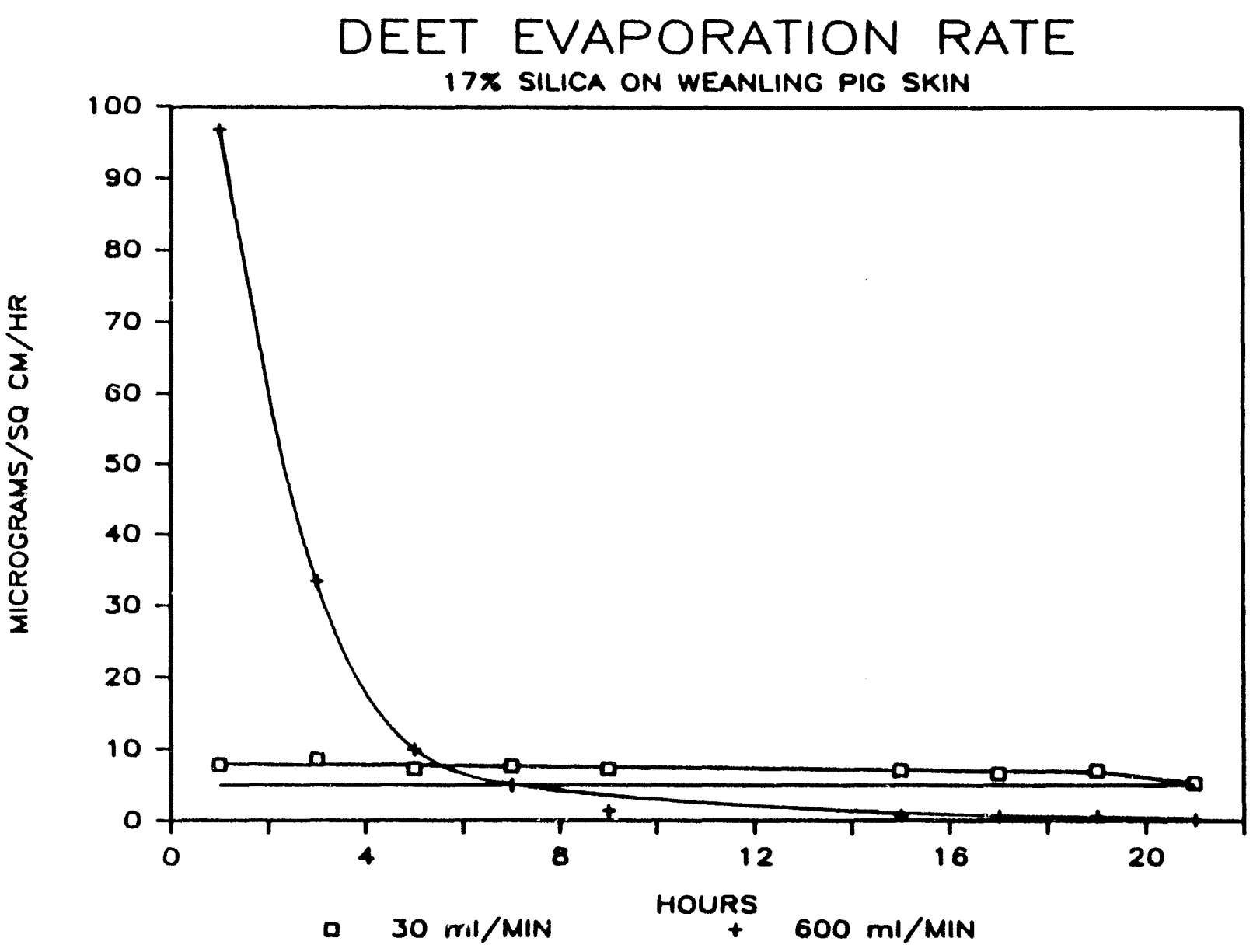

DEET EVAPORATION RATE
17% SILICA ON WEANLING PIG SKIN
MICROGRAMS/SQ CM/HR
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
30 ml/MIN
600 ml/MIN

Figure 20

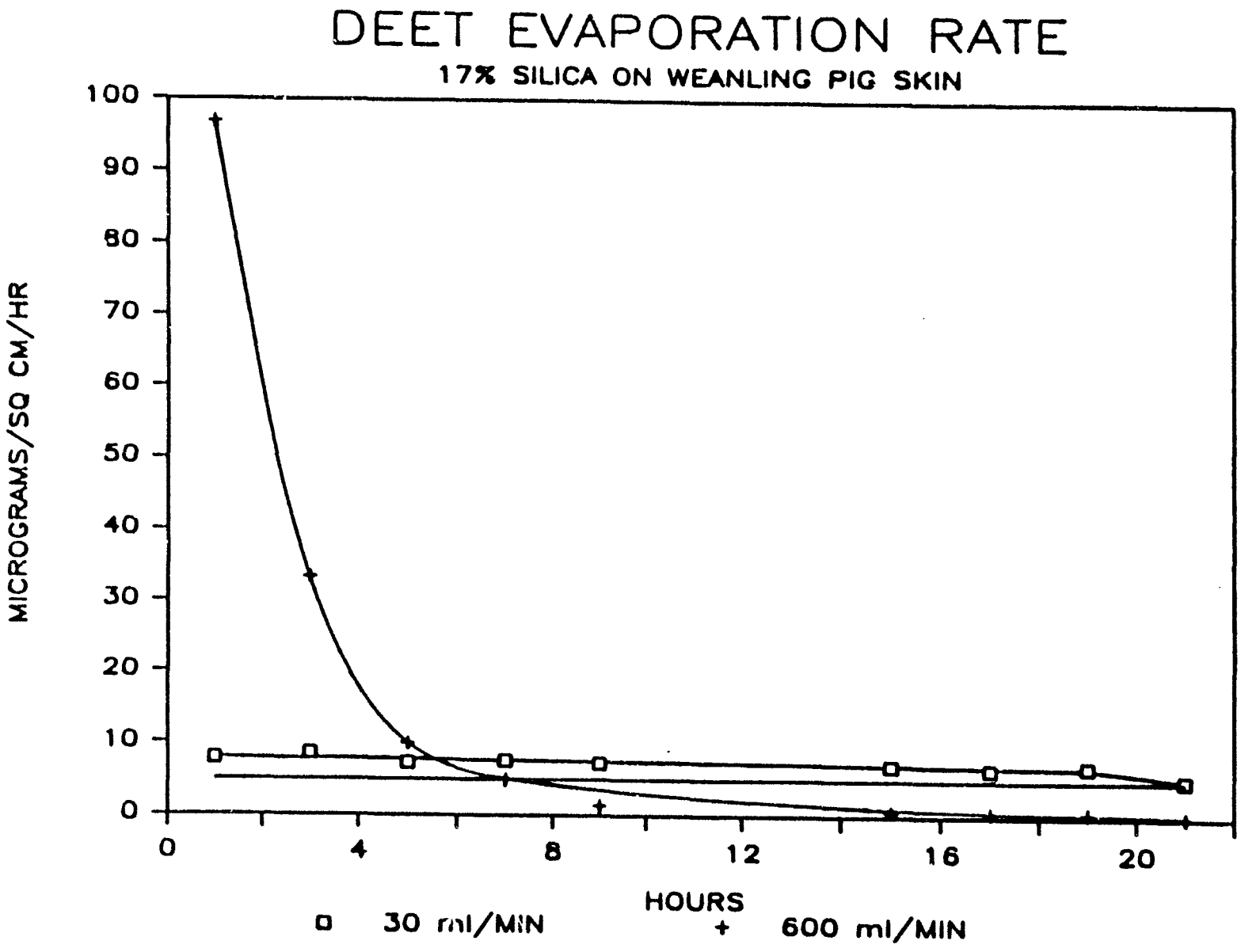
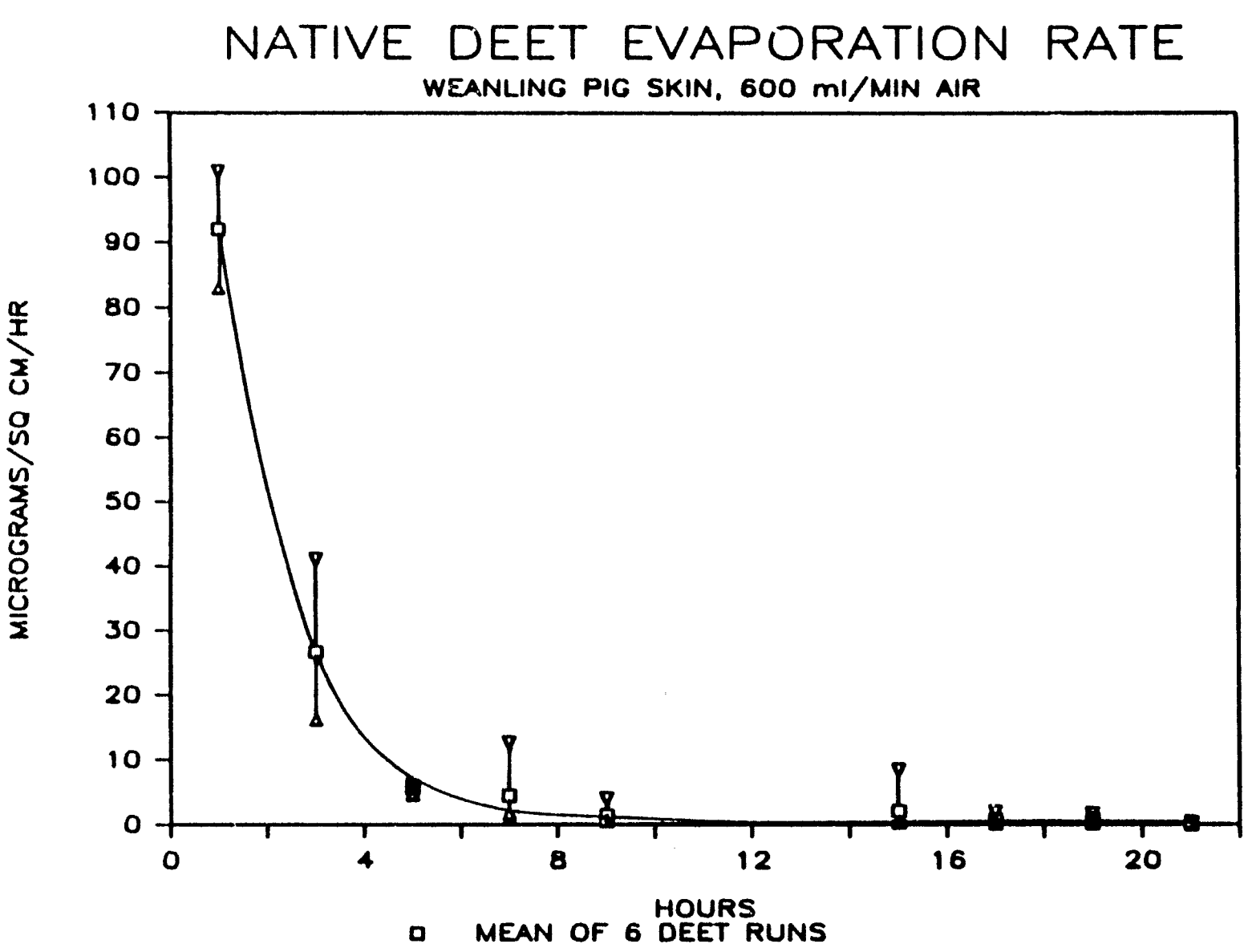

DEET EVAPORATION RATE
17% SILICA ON WEANLING PIG SKIN
MICROGRAMS/SQ CM/HR
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
30 ml/MIN
600 ml/MIN

Figure 21


NATIVE DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
MEAN OF 6 DEET RUNS

tively, the mean initial rate of Deet evaporation is two-fold higher in our studies than in Reifenrath's reports. Similarly, their duration of effective evaporation is ten hours or more, while we observe only five hours. While these quantitative differences cannot be explained, it is interesting that our duration closely approximates the in vivo duration of neet Deet protection of four to five hours (Reifenrath and Robinson, (1982); Reifenrath, Personal Communication).

4) Deet/Silica Type B Sustained Release Weight Ratios II

Next, evaporation profiles of four different Deet/silica mixtures were tested on the Reifenrath apparatus at the recommended air flow rate of 600 ml/min. The mixtures tested were native Deet, 17% silica, 25% silica, 50% silica, and 75% silica, all applied to split thickness pig skin at a dose of approximately 320 ug/cm^2.

As shown in Figure 22, the lower silica concentrations of 17 and 25 percent show few significant differences from the neet Deet evaporation profile. With increasing silica concentrations of 50 and 75 percent (Figure 23), the minimum effective evaporation rate of 5 ug/cm^2/hr was exceeded for a longer period of time; approximately 12 to 14 hours, as compared to neet Deet; 5 hours. Although the evaporation profiles show some variability, the overall trend is to an increasing length of time above the minimum effective evaporation rate at higher silica concentrations. Qualitatively, these data are quite similar to those obtained with hairless mouse skin and the 30 ml/min flow rate.

5) Deet/Hydrophobic Silica Gel Sustained Release Matrix

To further investigate the sustained release properties of Deet absorbed on silica gel, a preparation of a hydrophobic silica gel (HSG) was loaded with ^{14}C Deet at 75% and 50% (W/W) Deet and applied to the evaporation/penetration cell substrate at a dose of 320 ug Deet/cm^2. The hydrophobic silica gel has an average particle diameter of 0.2 microns. The evaporation rate of this matrix (Figure 24) was maintained at or above the critical level for 11 hours using the 1:1 ratio. Observing the 3:1 Deet/silica gel samples, only the 17-hour point was below the critical level. Increasing the hydrophobic nature of the silica gel increased the observed effective duration.

6) Deet/Silica Type B Sustained Release Matrices with Polymers

Silica gel Type B was treated with poly-L(-)lactide, polyvinyl pyrrolidone (PVP), and silicone and then loaded with ^{14}C Deet at a ratio of 50% silica gel/50% Deet. The final concentration of lactide polymer was 5%; that of PVP, 5%; and that of silicone, 1%. The materials were applied in duplicate to pig skin at a Deet dose of 320 ug/cm^2. As shown in Figure 25, silicone increased the duration of evaporation only slightly. With 5% PVP, evaporation rate was maintained above the critical level for approximately seven hours. The 5% lactide material maintained effective levels of evaporation for 13 hours. Again, these observations are qualitatively similar to those obtained previously with hairless mouse skin at a lower air flow rate.

Figure 22

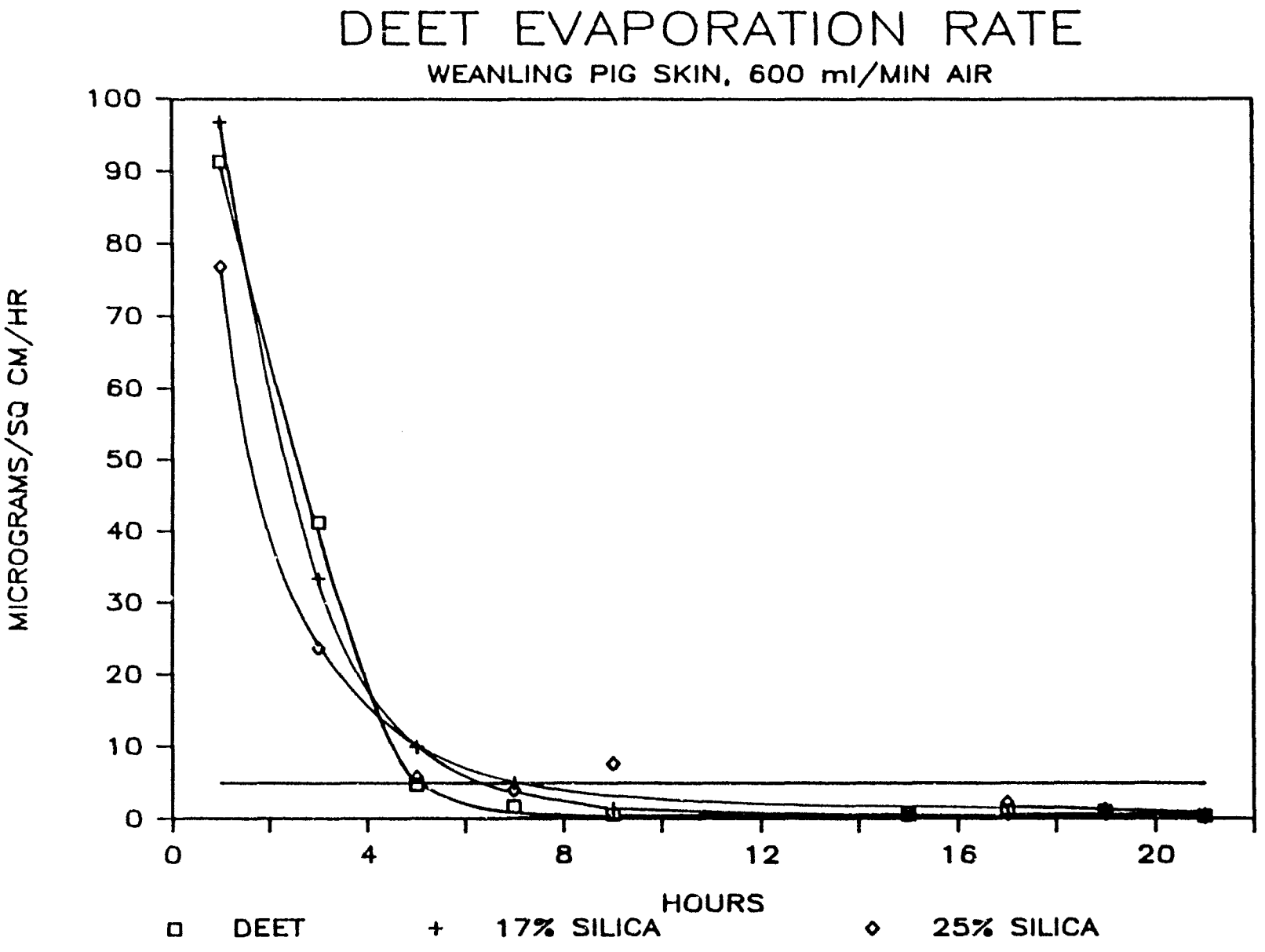

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
17% SILICA
25% SILICA

Figure 23

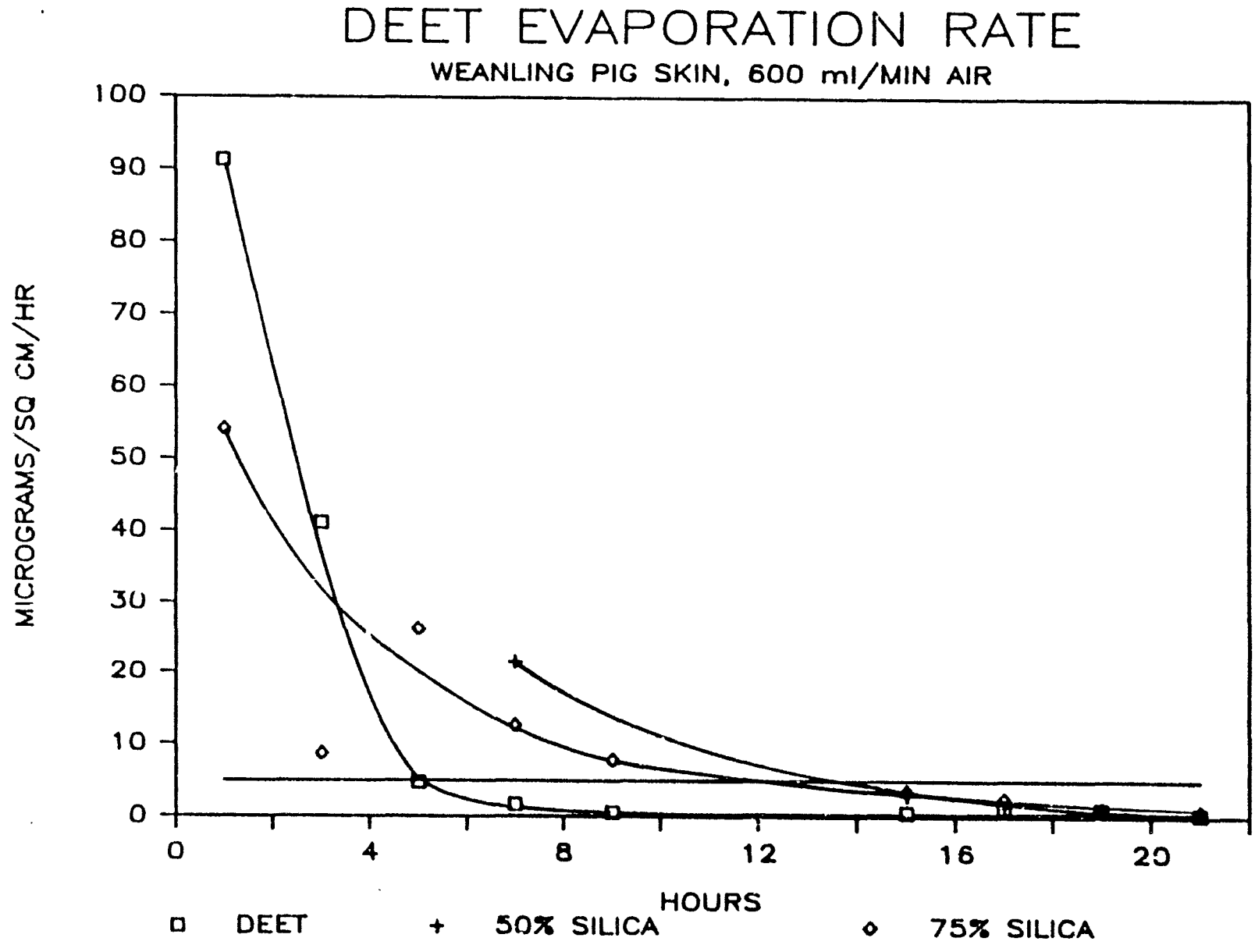

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
50% SILICA
75% SILICA

Figure 24

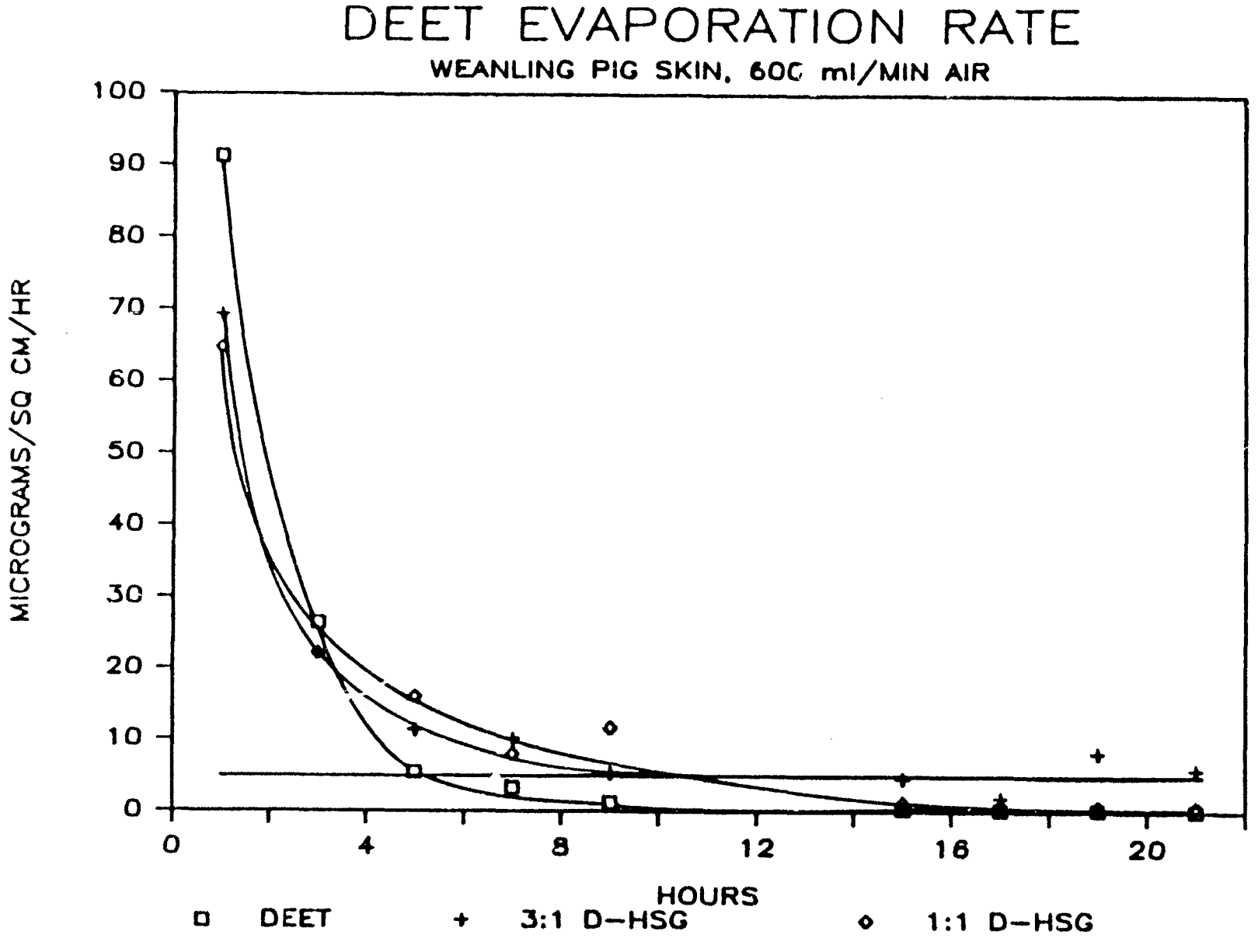
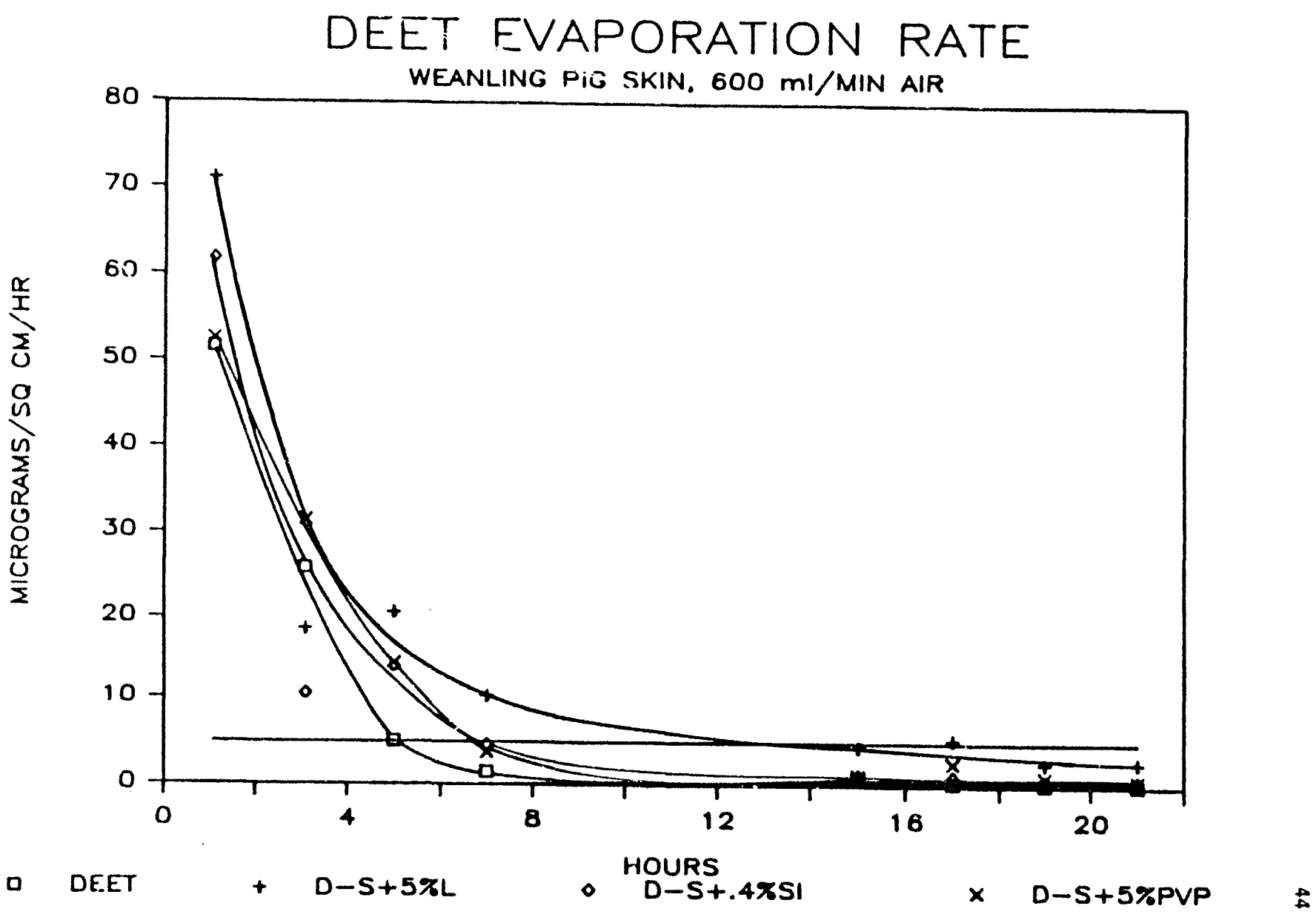

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
3:1 D-HSG
1:1 D-HSG

Figure 25


DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
D-S+5%L
D-S+.4%SI
D-S+5%PVP

Based on previous experiments, 1% PEG was added to the lactide-treated silica gel, and a 320 ug/cm² dose was applied to the test system. As shown in Figure 26, the addition of PEG slightly reduced the duration of effective evaporation to approximately ten hours rather than the 13 hours previously observed.

Silica gel/lactide powder was coated with approximately 1% poly-L(-)-lactide using a fluidized bed air suspension process. The resulting microcapsules were loaded with ^{14}C Deet and applied to pig skin at a dose of 312 ug/cm². The microcapsules released Deet at or above the critical level for approximately ten hours (Figure 27), not an improvement over uncoated particles.

7) Summary of Deet/Silica Sustained Release

The results of these in vitro studies are summarized in Table 6. The greatest increase in the duration of effective evaporation rate was observed with the hydrophobic silica gel; nearly five times the duration of neet Deet. An increase of two to three-fold was observed with Deet absorbed on silica gel alone or silica gel treated with polytactide.

8) Formulation Development

a) Aqueous Based Lotion

Two Deet/silica mixtures were selected for development into formulations; 2:1 Deet/silica and 3:1 Deet/hydrophobic silica (HSG). Each was formulated with a water based emulsion cream base, Formula 2968-19A, supplied by H. Y. Shuster, Inc., (see Appendix II) at a ratio to provide 15% active ingredient (Deet) concentration in each test formulation.

These water based formulations were run in a total of six cells each for 22 hours with no conclusive results. Total recoveries for the Deet/silica aqueous lotion formulation ranged from 24% to 68%, while recoveries ranged from 18% to 71% for the Deet/HSG aqueous lotion formulation. Plots of the evaporation curves for two runs for each formulation are shown in Figures 28 and 29. The large discrepancy between replicate sample evaporation profiles indicated a homogeneity problem.

Because of the wide range in recoveries and evaporation profiles, an experiment was performed to determine if the formulations were homogenous. Several 3 microliter samples of each ^{14}C labeled formulation were homogenized, pipetted into scintillation vials and counted in 15 mls of NEN Bioflour to determine homogeneity. Results are shown in Table 7.

The test data show that, while each of the native Deet samples contained a consistent concentration of Deet, the hydrophobic silica gel/Deet aqueous lotion mixtures and silica gel/Deet/aqueous lotion mixtures contained a wide range of Deet concentrations. This effectively shows the nonhomogeneity of the Deet/silica/aqueous lotion blends.

Through the above data and visual observations, it was determined that the water based lotion and Deet were incompatible; the water based emulsion either broke down in the presence of Deet, or water replaced the Deet in the silica, producing a nonhomogenous solution.

Figure 26

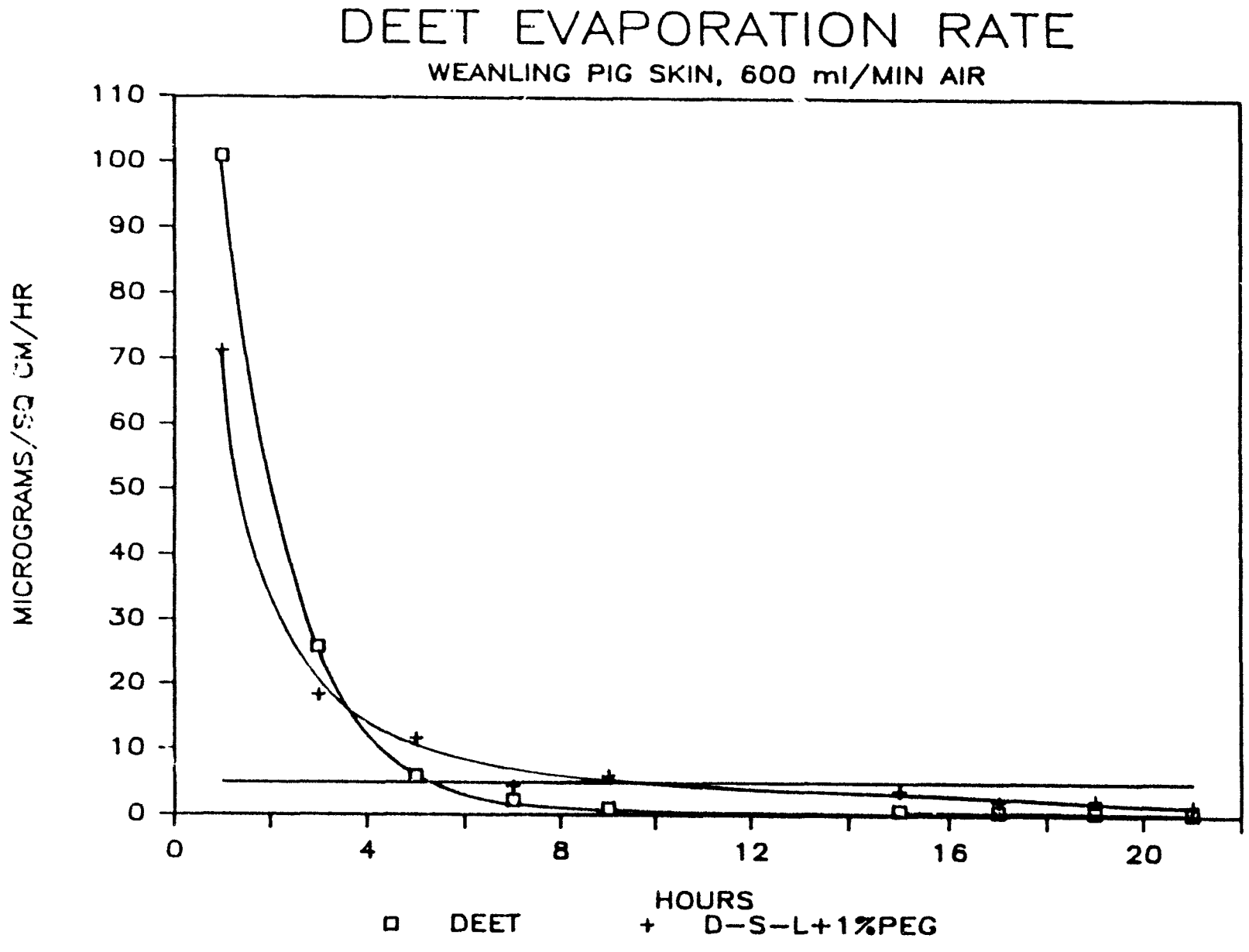

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
D-S-L+1%PEG

Figure 27

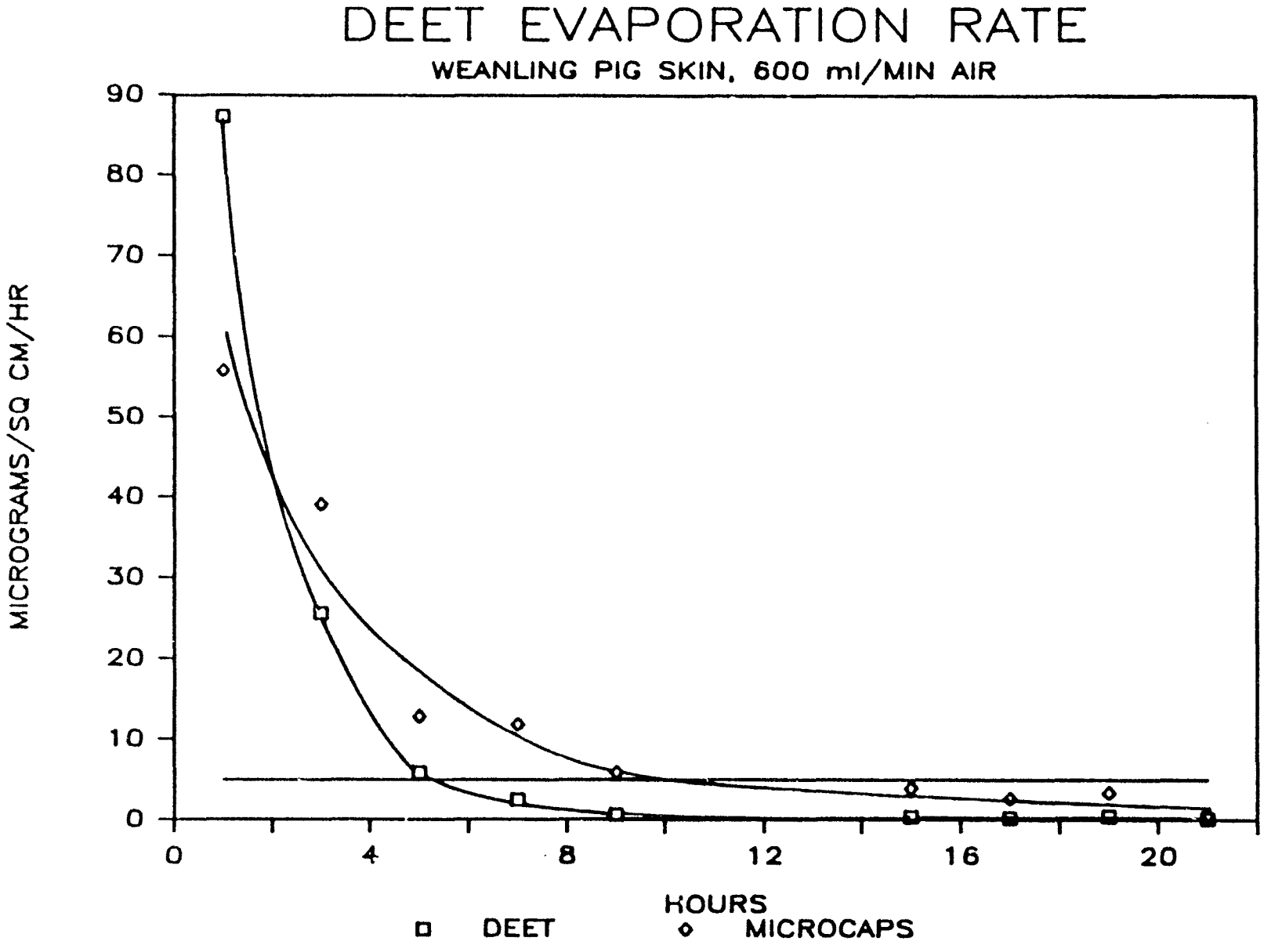

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
DEET
MICROCAPS

TABLE 6

DURATION OF DEET EFFECTIVE EVAPORATION

FROM WEANLING PIG SKIN

No.	Test Material	Duration* Hours
1	Neet Deet	5
2	1:1 Deet Silica	14
3	1:3 Deet Silica	12
4	1:1 Deet Silica + 5% Polylactide	13
5	1:1 Deet Silica + 5% Polylactide + 1% PEG	10
6	3:1 Deet Hydrophobic Silica	24
7	1:1 Deet Silica + 1% Polylactide Coating	10

* Hours at levels greater than 5 ug/sq. cm./hr as suggested by Dr. Reifenrath.

Figure 28

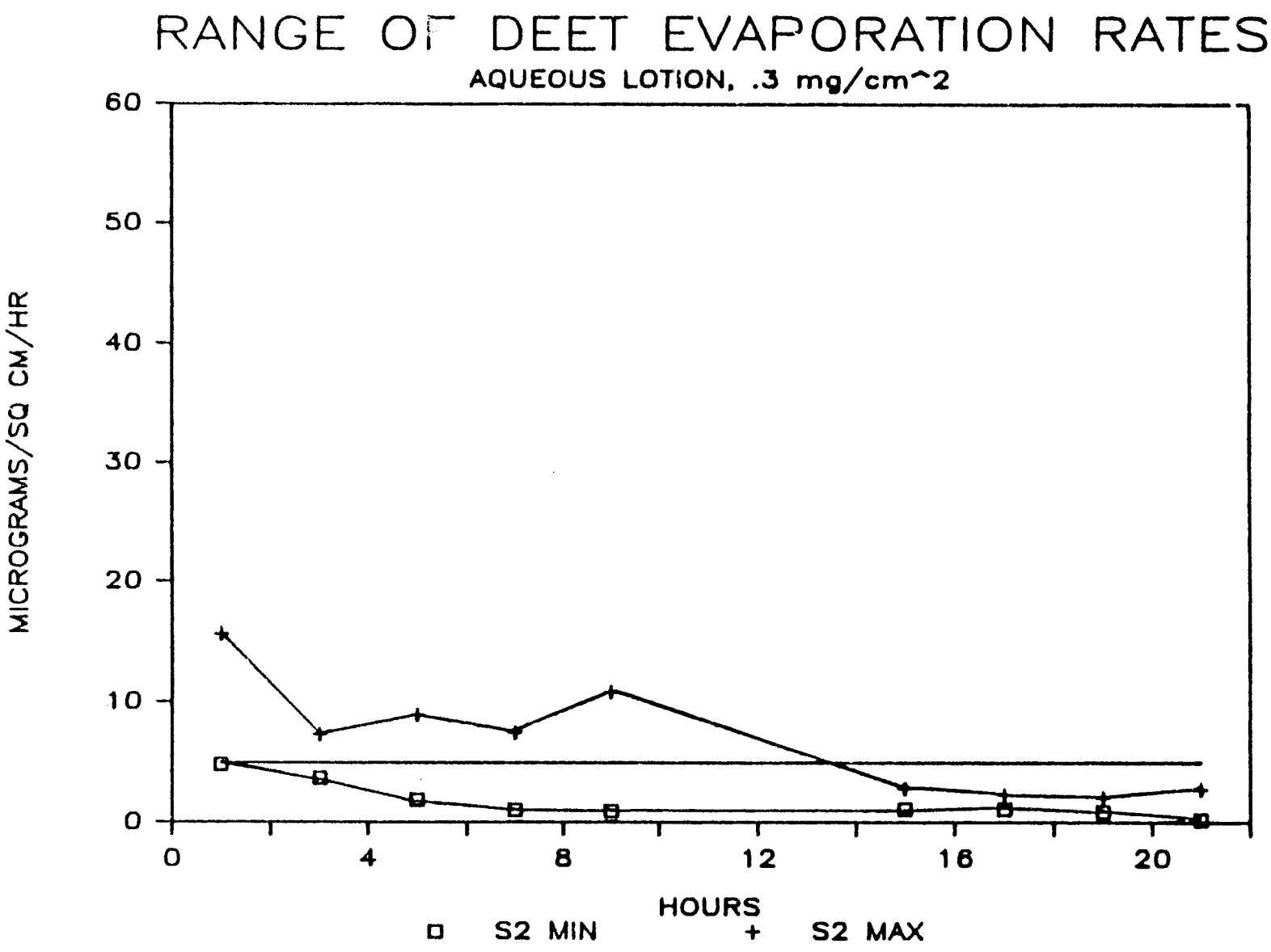

RANGE OF DEET EVAPORATION RATES
AQUEOUS LOTION, .3 mg/cm^2
MICROGRAMS/SQ CM/HR
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
S2 MIN
S2 MAX

Figure 29

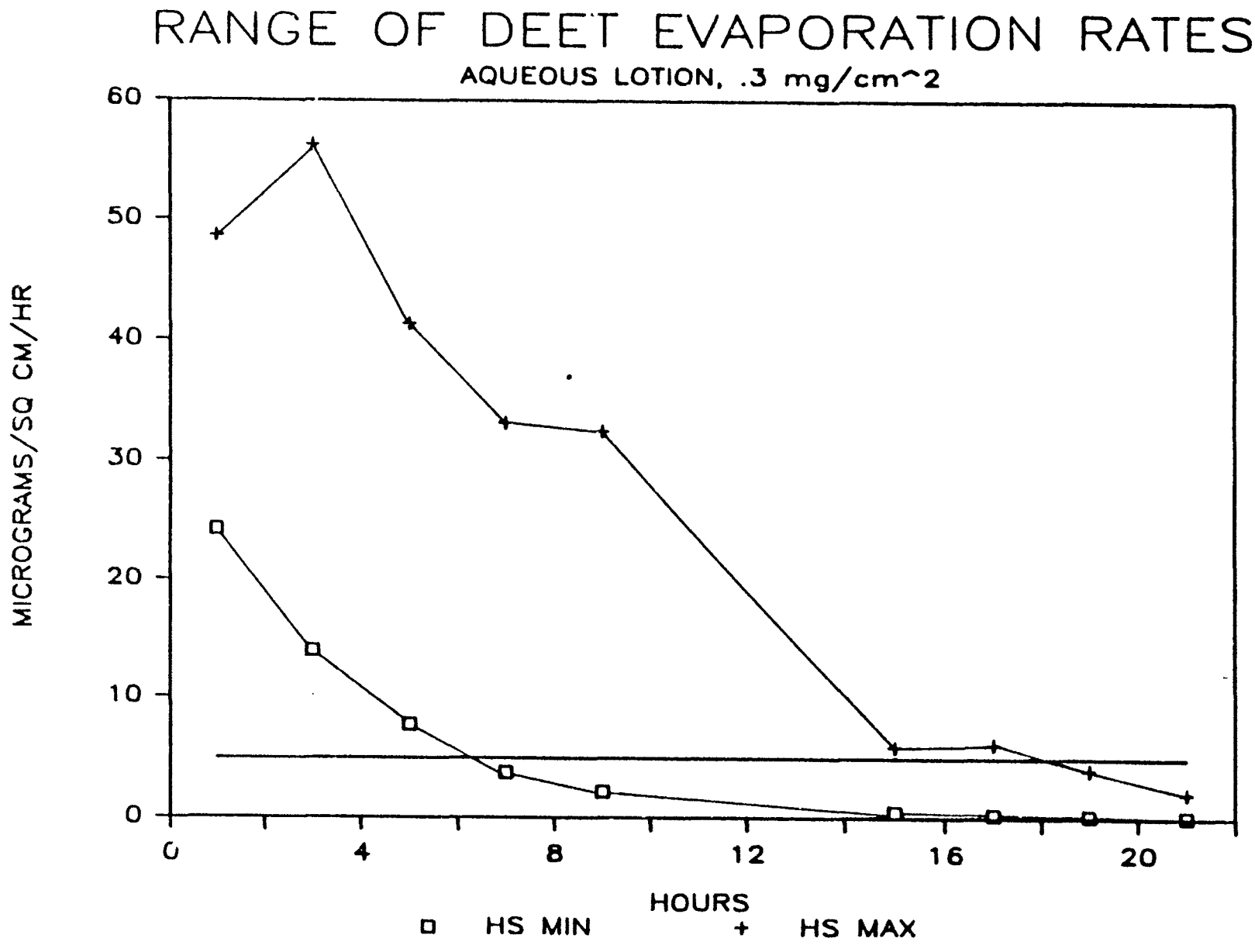

RANGE OF DEET EVAPORATION RATES
AQUEOUS LOTION, .3 mg/cm^2
MICROGRAMS/SQ CM/HR
60
50
40
30
20
10
0
0
4
8
12
16
20
HOURS
HS MIN
HS MAX

Table 7

Aqueous Lotion Standards

Sample	Volume (Microliters)	CPM	DEET (mg)	DEET % Concentration
MD 1	3	94123	.501	16.7
MD 2	3	95020	.505	16.8
HS Aqueous Lotion 1	3	50508	.274	9.1
HS Aqueous Lotion 2	3	36985	.201	6.7
HS Aqueous Lotion 3	3	82801	.450	15.0
S Aqueous Lotion 1	3	56210	.304	10.1
S Aqueous Lotion 2	3	49103	.265	8.8
S Aqueous Lotion 3	3	54967	.297	9.9

b, Alcohol Based Lotion

To alieviate this problem, an alcohol based lotion was developed by H. Y. Shuster, Inc., Formula 3002-42B (see Appendix II) which allowed a homogenous, stable formulation. Two alcohol based lotion formulations were prepared; one containing 2:1 Deet/SG mixture and the other containing the 3:1 Deet/HSG mixture. Each was blended at a 40% Deet mixture and 60% alcohol lotion weight ratio. To ascertain if these blends were homogenous, more standards were taken and the Deet concentration determined. The results are in Table 8. As can be observed from the data, these alcohol based lotions are homogenous. In the first test of these new formulations on the Reifenrath apparatus, a dose of 300 mg/cm² Deet was applied to the weanling pig skin substrate. As a control, 300 mg/cm² of 40% native Deet/60% alcohol lotion base was also run to determine the effect of the lotion base alone on Deet evaporation.

The resulting evaporation curves are shown in Figure 30. Native Deet/lotion evaporation above the Minimum Effective Evaporation Rate (MEER) is extended from four hours to almost seven hours, while the Deet/SG and Deet/HSG lotion formulations remained above the MEER for 11 and 12 hours, respectively.

Three alcohol lotion based formulations were prepared for in vivo efficacy, troop acceptability, toxicity, and materials compatibility testing. The formulation containing 2:1 Deet/silica Type B was labeled HS-30. The two formulations containing differing amounts of 3:1 Deet/HSG were labeled HSL-44 and HSL-50.

HSL-44 was selected as our final product on the basis of the above tests.

9) HSL-44 Sustained Release Evaporation Profile

The last in vitro release study was done using BIOTEK's final formulation containing 44% active ingredient, Deet. This was applied to weanling pig skin substrate at a dose of 2 ul (approximately 2 mg) per square centimeter; an average application of arthropod repellent on exposed skin. Six evaporation/penetration chambers were prepared and started. One substrate ruptured during the course of the experiment, leaving five complete throughout the run.

The mean evaporation rate of this formulation at 2 ul/cm²is shown in Figure 31. The evaporation rate is initially 87 ug/cm²/hr, and remains above the minimum effective evaporatior rate, 5 ug/cm² /hr, for 22 hours. The mean Deet penetration rate is shown i Figure 32.

At the conclusion of the 24-hour experiment, 71.4% ± 9.0% of the Deet had evaporated, 12.1% ± 5.2% had penetrated through the skin, and 17.6% ± 4.4% remained either on the surface of the substrate, in the substrate, or on the internal walls of the evaporation cell.

A summary of the disposition of radioactivity after 24 hours is shown in Table 9.

Table 8

Alcohol Lotion Standards

Sample	Volume (µl)	CPM	DEET (mg)	DEET %
HS Alcohol Lotion 1	10	84451	.604	6.0
HS Alcohol Lotion 2	10	85510	.612	6.1
S Alcohol Lotion 1	10	90884	.635	6.4
S Alcohol Lotion 2	10	91679	.641	6.4

Figure 30

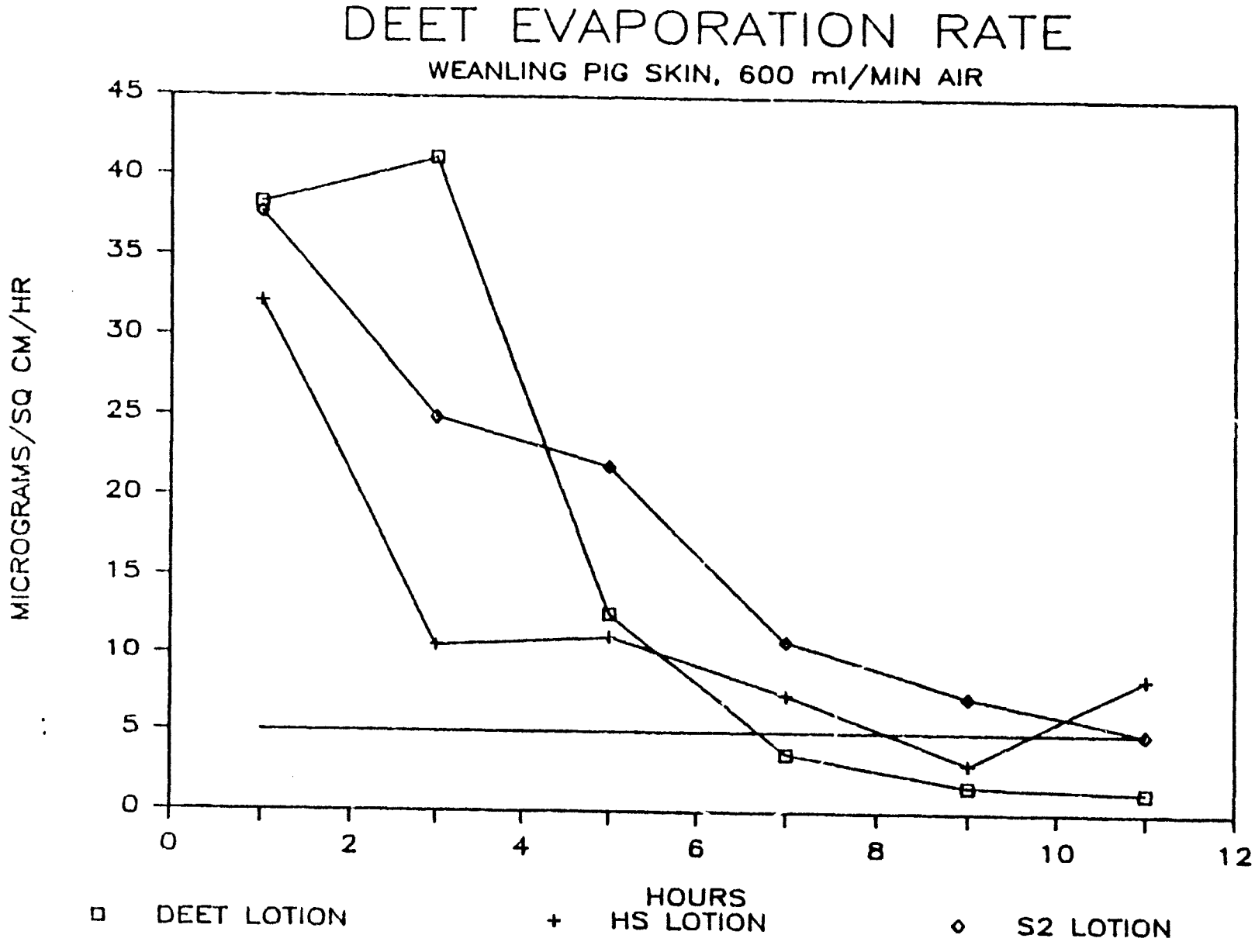

DEET EVAPORATION RATE
WEANLING PIG SKIN, 600 ml/MIN AIR
MICROGRAMS/SQ CM/HR
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
HOURS
DEET LOTION
HS LOTION
S2 LOTION

Figure 31

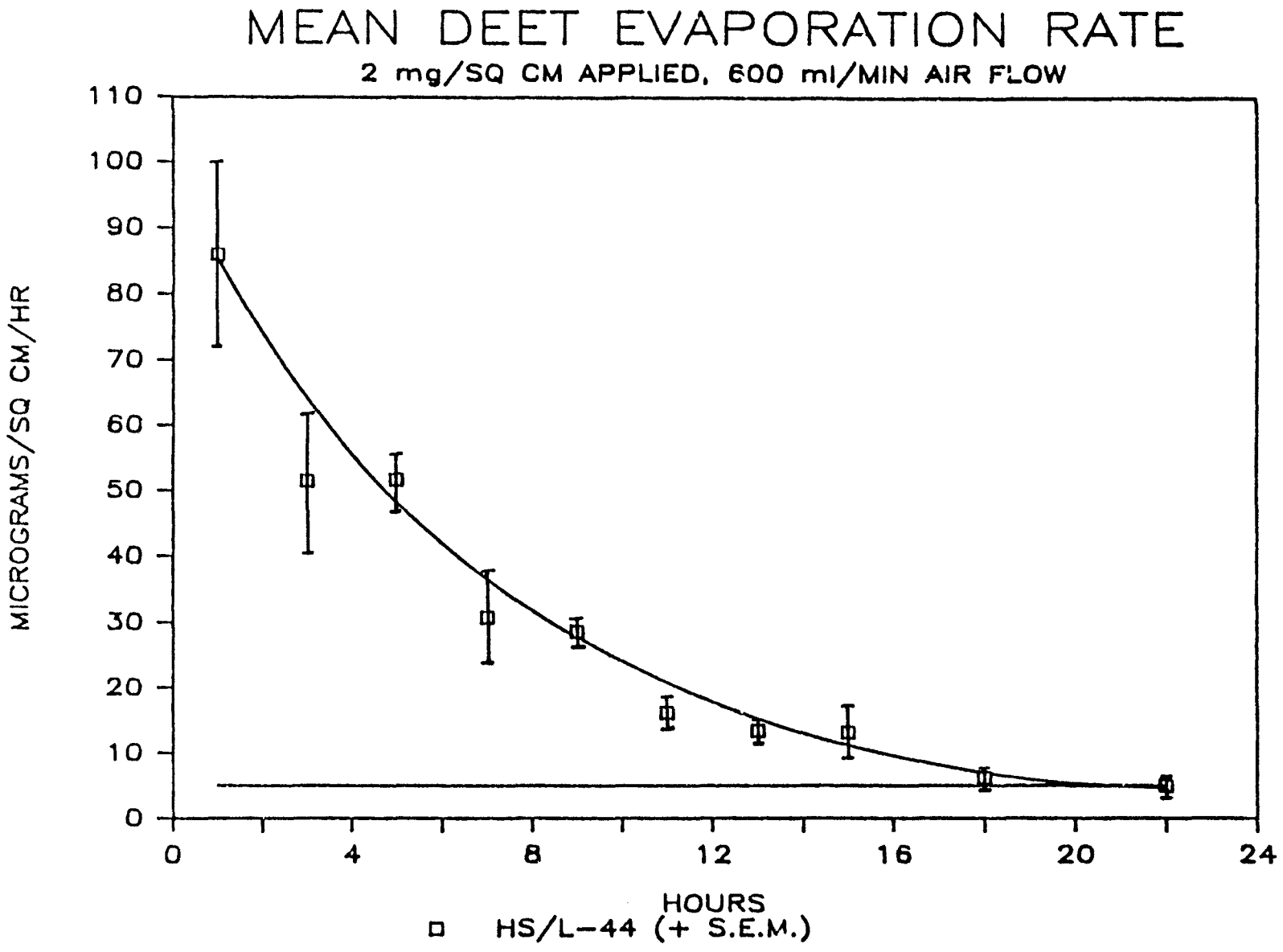

MEAN DEET EVAPORATION RATE
2 mg/SQ CM APPLIED, 600 ml/MIN AIR FLOW
MICROGRAMS/SQ CM/HR
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS
HS/L-44 (+ S.E.M.)

Figure 32

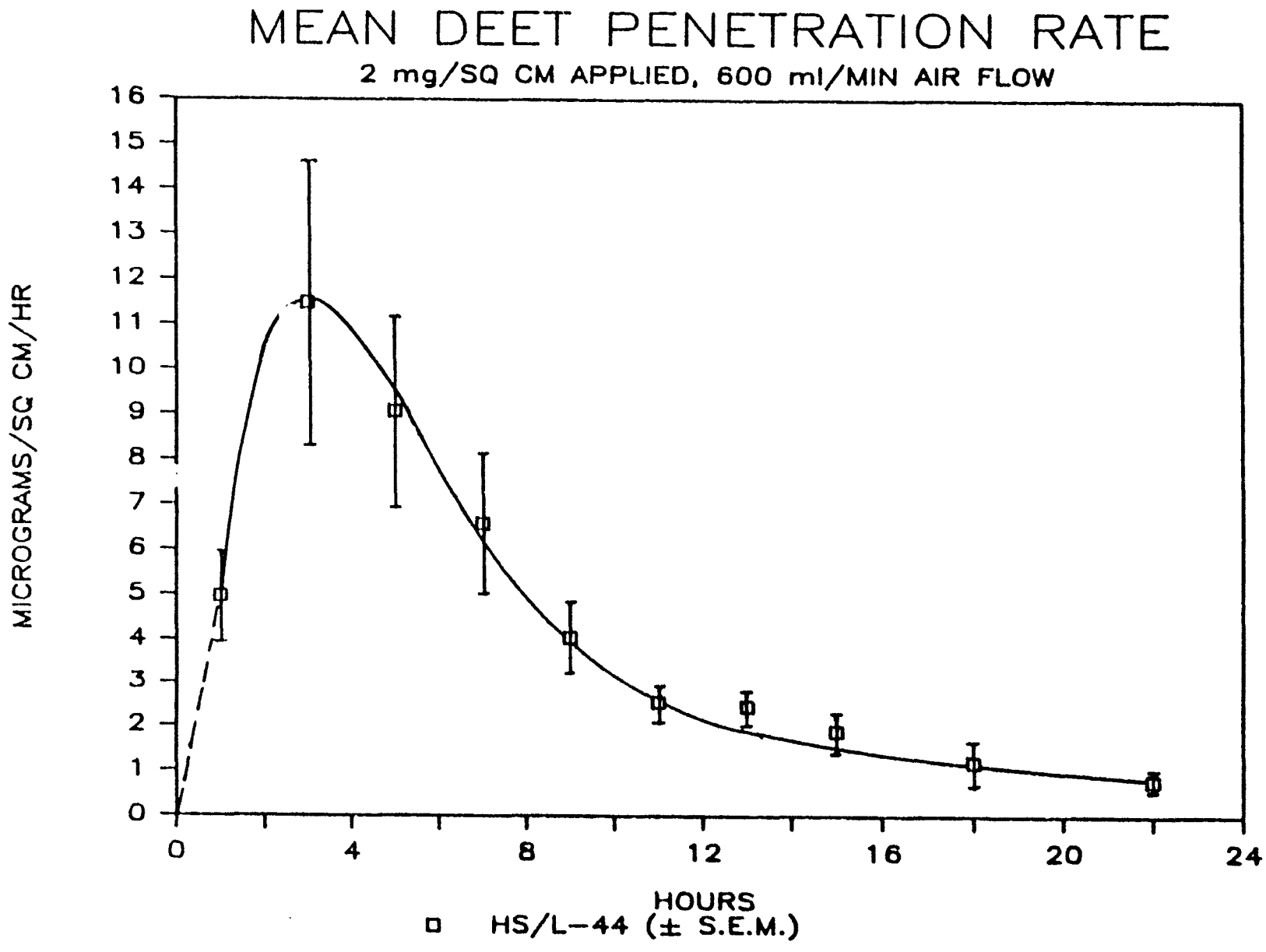

MEAN DEET PENETRATION RATE
2 mg/SQ CM APPLIED, 600 ml/MIN AIR FLOW
MICROGRAMS/SQ CM/HR
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
HOURS
HS/L-44 (± S.E.M.)

Table 9

Disposition of radioactivity 24 hours after application of radiolabeled HSL-44 to weanling pig skin at a dose of 2 mg/cm^2. Air flow rate of 600 ml/min.

	Percent of Applied Dose				
Cell	Evaporation	Percutaneous Penetration	Skin	Rinse	Total Recovery
1	57.5	9.8	16.8	2.8	86.9
2	74.9	17.1	11.5	0.5	104.0
3	68.3	17.6	15.2	0.8	101.9
4	75.1	11.0	20.0	0.6	106.7
5	81.3	5.2	19.3	0.2	106.0
Average	71.4±9.0	12.1±5.	16.6±3.4	1.0±1.0	101.1±8.2

Averages calculated as the mean ± S.E.M.

$$\text{S.E.M.} = \sqrt{\frac{n \Sigma (X^2) - \Sigma (X)^2}{n (n-1)}}$$

C. *In Vivo* Efficacy Studies

1. Methodology

a. Rearing and Handling of Mosquitoes

Aedes aegypti mosquitoes were reared in a specially designed 300 square foot insectary located in Dr. Andrew Spielman's laboratory at the Harvard School of Public Health. The room is subdivided into four cubicles. Temperature was regulated between 24 and 25°C; humidity ranged from 73 to 77%; and the space was lighted for 16 hours each day with 40-minute crepuscular periods. Eggs were hatched in distilled water in glass-covered enamel pans; larvae were fed Purina™ Guinea Pig Chow and adults maintained on 10% sucrose. Each day, pupae that formed were harvested by gentle sieving and transferred in lots of 50 to distilled water in 1-gallon polycarbonate, screen-topped containers. Two days after emergence, the adult mosquitoes were transferred by aspirator to similar containers with the water contained in 200 ml vessels previously placed within the large containers. Conditions were regulated to produce well-nourished adults with wings 2.8 - 3.0 mm in length. Dacron pledgets soaked with 10% sucrose solution were placed on the screen top of each container and renewed daily. At four to six days of age, mosquitoes were placed in a 5°C cold room until motionless. The females were segregated and transferred to test chambers. Each test chamber contained 20 mosquitoes. Mosquitoes were kept at 5°C for no more than 30 minutes and were handled only by the hind legs with soft-tipped forceps. In order to test the effect of cooling on subsequent behavior, several test chambers were loaded at 20 - 25°C by aspirating active mosquitoes. No differences in behavior were observed.

For transport, loaded test chambers were placed in insulated chests. Transit time to the testing site, Herbert V. Shuster, Inc., Quincy, MA, did not exceed thirty minutes. Following arrival of the containers at the testing facility, the mosquitoes were acclimatized to room temperature and light conditions for at least two hours.

b. Protocol

Efficacy of the sustained action arthopod repellent was determined in laboratory tests. Subjects were males of military age, 18 to 36 years, who had not shown a sensitivity to insect bites or other repellents. Informed consent was obtained from each subject (see Appendix II). Testing was conducted at the laboratories of H. V. Shuster, Inc., Quincy, MA and was supervised by both BIOTEK and Shuster employees. The efficacy testing protocol, which was available to all subjects, is presented in Appendix II.

On the morning of the experiment, subject forearms were washed with a natural soap containing no perfume, deodorant, or other cosmetic enhancers, and rinsed with 70% ethanol. For each subject control site, (5 cm in diameter), and two test sites, 6 x 16.7 cm rectangles were outlined on the ventral forearms with a waterproof pen. Locations of the control and test sites and treatment assignments were selected using a random number table. Test material was applied at a dose of 2 mg of formulation per square cm, spread evenly over the site, and the time recorded. Equipment used in *in vivo* procedures is listed in Appendix I.

Repellency was tested at two-hour intervals for six tests (i.e., 2, 4, 6, 8, 10, and 12 hours following application). To minimize subject discomfort, one test interval for each subject was selected by a random number table previously generated to include tests of repellent test sites and the control site. For other intervals, only repellent test sites were tested. For each test, subjects were comfortably seated with forearms supported on a table in the testing laboratory. As shown in Table 10, the temperature of the testing laboratory, which was determined at each test interval, was reasonably constant during each test session and between test sessions. Humidity levels were considerably higher than the 50% desired and showed significant variability. Light levels were determined with a Gossen Luna-Pro light meter and were constant at 350 Lux. Temperature and humidity were measured with a sling psychrometer (Bacharach, Pittsburgh, PA).

For each test, a mosquito test chamber (Figure 33) containing approximately 20 mosquitoes was placed on the appropriate site, and mosquito behavior observed by at least two people. Mosquitoes had access to the subjects' skin through the mosquito netting for a period of two minutes, after which the chambers were removed from the forearms. The mosquitoes were destroyed and, to prevent any carry-over of repellent, the mosquito netting was replaced and the chambers washed before the next use. At the completion of the two-minute observation period, the number of mosquitoes feeding was recorded.

In order to determine compatibility of the formulations with camouflage face paint, face paint was applied to one randomly chosen test site for each subject. Repellent formulation was then applied over the face paint and to an uncamouflaged test site and efficacy tests were conducted as described.

Efficacy was determined under ambient laboratory conditions and three conditions which are modifications of the Climate Design Types described in AR 70-38: hot-humid, variable high humidity, and basic-hot. Maximum temperature and humidity changes were compressed into the 12-hour test period. The objective was to increase temperature slowly so that temperature was maximized at the end of the fifth hour of the experiment, maintained for one hour, and then slowly decreased to reach the initial value at the beginning of the eleventh hour. The parameters for each condition are presented in Table 11. Following application of the repellent formulation, subjects entered the environmental chamber (12 x 9 x 9 feet) and climatic condition temperature and humidity modifications were instituted. Temperature and humidity were monitored at hourly intervals. Since mosquito feeding behavior is dependent on environmental conditions, subjects returned to the testing laboratory for efficacy testing at ambient conditions and reentered the environmental chamber at the conclusion of each observation period.

c. Apparatus

Test Cage - The mosquito cages used for the in vivo efficacy tests were designed as a modified version of the World Health Organization (WHO) test chambers. The test cage is clear acrylic plastic, cylindrical, 5 1/2" long, 1 3/4" internal diameter, and 2.0" outside diameter. One end of the cylinder is sealed with a 2" x 2" x 1/8" piece of clear acrylic. A 7/8" diameter circular opening is drilled in the center of the sealed end and fitted with a No. 4

TABLE 10

MOSQUITO EFFICACY TEST
TEST ENVIRONMENT

TEST SESSION	TEMPERATURE (°C)	HUMIDITY REL. %
I	24.6±0.6	63.4±1.3
II	24.4±0.3	68.5±1.2
III	24.8±0.4	72.8±1.6
IV	25.2±0.1	66.3±1.9
V	23.2±0.3	66.0±0.9

Data are presented as means ± S.E.M.

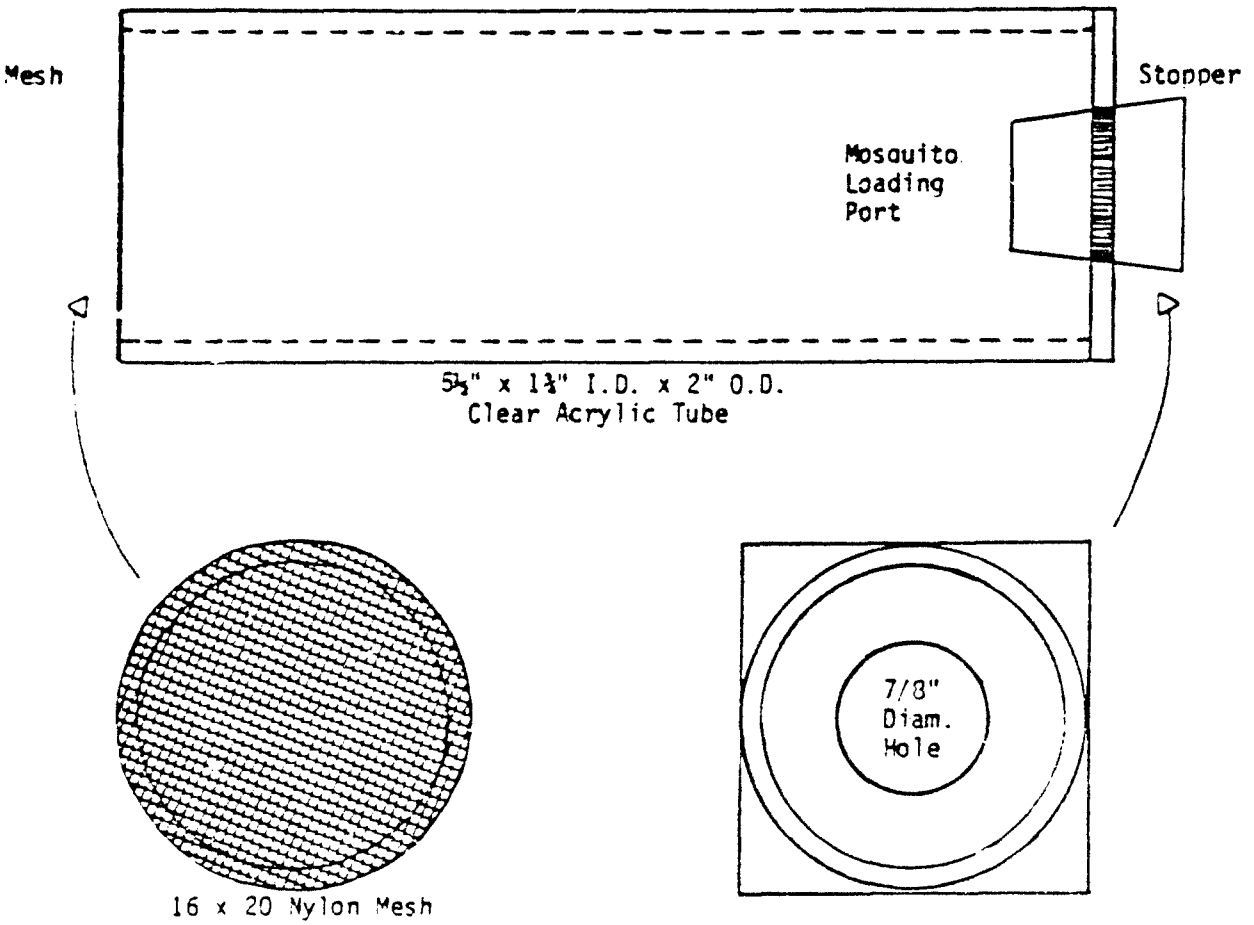
Table 11

Prescribed Climatic Conditions
AR-70-38

	Initial		Maximum	
Condition	Temperature	Relative Humidity	Temperature	Relative Humidity
Hot-humid	31°C	88%	41°C	52%
Variable high/ humidity	26°C	100%	35°C	62%
Basic-hot	30°C	44%	43°C	22%

Figure 33

IN VIVO EFFICACY TEST MOSQUITO CHAMBER



stopper for insertion of the mosquitoes. The other end of the cylinder is sealed with a 2" diameter piece of nylon mosquito netting, 16 x 18 mesh, glued in place. A diagram of the test chamber is shown in Figure 33.

Template - The template is a 6 x 16.7 piece of clear acrylic plastic, 1/8" thick. This was used as a guide in outlining the area where the repellent was applied.

d. Efficacy Data Analysis

Efficacy was quantitated by two measures, percent repellency and complete protection time. Percent repellency was calculated as follows:

$$\% \text{ Repellency} = 1 - ((\text{test bites}/\text{control factor})\ (1/n)) \times 100$$

where test bites = number of bites in a test

n = number of mosquitoes in test chamber

control factor = mean value of $\frac{\text{number of control bites}}{n}$ for that day

Percent repellency values less than zero were recorded as zero. For each subject, the percent repellency was calculated at each test session, and the mean value calculated for each test session. The complete protection time, CPT, was defined as the time during the test session at which two or more bites occurred. This is equivalent to the definitions in ASTM E939-83 and the Johnson draft for a proposed new ASTM procedure for laboratory tests of repellency (personal communication). This definition allows any number of single (unconfirmed) bites. The measure is less affected by a single extreme value than percent repellency when the number of subjects is small.

2. In Vivo Experiments and Results

Pilot efficacy studies were conducted to establish the optimum experimental parameters. Several varieties of test chamber were considered. A chamber with a floor area to biting port area ratio similar to that of the chamber described in ASTM E951-83 gives a low biting pressure. A modification of the World Health Organization (W.H.O.) chamber, which is essentially a tube with a nylon net at one end and a biting port at the other with a movable slide to expose the skin, was tested. Good biting behavior was observed. However, at the end of the test all of the mosquitoes could not easily be dislodged into the tube before closing the slide, and several escaped. The chamber was reversed and the nylon covered end was placed on the skin. The nylon did not interfere with appetative behavior. It was noted that when the chamber was used in this manner on skin freshly treated with Deet formulations, subsequent use of the chamber with naive mosquitoes gave no bites. Apparently Deet remaining on the nylon was repelling the insects. Therefore, fresh nylon netting was used in subsequent experiments. For reasons of economy and ease of use the test chamber selected consisted of a 5 1/2-inch section of 1 3/4" I. D. x 2" O.D. Clear acrylic tube covered on one end with nylon mesh and a loading port on the opposite end closed with a rubber stopper (Figure 33).

Using a test chamber of this design in an air conditioned laboratory at approximately 20°C and 50% relative humidity, 82% of the insects began feeding within 2 minutes and none had fed to repletion and left the site. Insects were

leaving the site after feeding for 2.5 minutes. The low biting response was probably a result of not restricting sugar water previous to the test. When mosquitoes were deprived of food and water for 18 to 24 hours previous to testing, 90.8% ± 2.3 S.E.M. (n = 6) of the mosquitoes were feeding 2 minutes after testing began. In these pilot studies no difference was observed in percent feeding response at the 2 minute time with from 10 to 30 mosquitoes per test chamber.

Based on these results, the number of mosquitoes available, and the number of tests required, the exposure time selected was 2 minutes, and each test chamber was to contain 20 female Aedes aegypti mosquitoes.

Biting pressure in the in vivo efficacy studies conducted in the laboratories of H.V. Shuster, Inc. was significantly lower ($p < 0.005$ by Student's t test) than that observed in the pilot studies, 70.0% ± 4.9 (S.E.M.) versus 90.8% ± 2.3 (S.E.M.). This was a potential problem since it effectively reduced the sensitivity of the assay. With 20 mosquitoes in each chamber each bite at 90.8% biting pressure is equivalent to 5.5% efficacy level while at 70.0% biting pressure each bite equals 7.1% efficacy level, a 30% increase. Several variables which may have reduced biting pressure were investigated. Increasing the period of food deprivation from 12 to 24 hours had no effect. No differences were observed in biting behavior between mosquitoes loaded into test chambers with an aspirator at room temperature and those loaded in an inactive state by cooling to 5°C. However, virtually 100% of the cooled mosquitoes survived the entire test period while up to 80% of the aspirated mosquitoes did not survive the 27 hours required. Allowing access to drinking water on paper towels and altering humidity during transportation from the rearing laboratory to the testing facility also did not appear to have a significant effect. The variable which remains is the transportation of mosquitoes in test chambers. Biting pressure was consistently higher in the rearing laboratory than at the testing facility.

The control data obtained during the in vivo efficacy study was analyzed to insure that bias was not introduced. Since control test periods were assigned in a random fashion, all cells were not equal. Therefore, one-way rather than two-way analysis of variance was utilized to test for effects of test session or test period (hour) on control biting response. There was no significant difference ($p > 0.11$) between test sessions (days) and between test periods (hours); there were also no significant differences ($p > 0.77$). Multiple range tests did not detect significant differences between groups at the 0.05 level. These analyses suggest that determination of control biting responses by a single test for each subject at a randomly selected time is appropriate.

During Test Sessions I and V (Table 10), repellent formulations were evaluated for efficacy under ambient laboratory conditions. Three formulations were tested, HSL-44, HSL-50, and SL-30. Formulations HSL-44 and HSL-50 were also evaluated for compatibility with camouflage face paint. The data is summarized in Table 12. In these tests two bites were suffered by HSL-44 treated subjects and one bite by HSL-50 treated subjects. In all cases the repellency was not significantly less than 100%. Similarly, the complete protection time (CPT) exceeded 12 hours in each experimental group. Camouflage face paint had no significant effect on repellent efficacy based on this test.

TABLE 12
REPELLENT EFFICACY*
AMBIENT CONDITIONS

TIME (HOURS)	HSL-44	HSL-44 CAM**	HSL-50	HSL-50 CAM	SL-30
2	100±0	100±0	100±0	100±0	100±0
4	100±0	100±0	100±0	100±0	100±0
6	100±0	100±0	100±0	100±0	100±0
8	100±0	100±0	100±0	100±0	100±0
10	100±0	100±0	99.0±1.0	100±0	100±0
12	97.0±3.0	100±0	100±0	100±0	100±0
CPT***	>12	>12	>12	>12	>12
n	5	5	10	5	5

* Data are presented as mean percent repellency ± S.E.M.
**CAM indicates efficacy when repellent was applied over camoflague face paint.
***CPT = Complete protection time

In the environmental chamber, temperature was more easily controlled than humidity (Table 13). Throughout the studies, light levels in the environmental chamber were maintained between 325 and 350 Lux. In the hot/humid condition, temperature was initially lower by 3° than required (Table 13). The required maximum temperature (41°C) was reached at the appropriate time (5 hours) and maintained for a full hour. The return to the initial temperature was more rapid than planned. In this condition, humidity was initially 5% below the desired level and, when adjustment was attempted, the mark was overshot by 6%. During most of the study, relative humidity was nearly 10% above the desired level. In the variable high humidity condition, temperature increased somewhat faster than desired and the maximum was 3° too high. Initial humidity levels were below the 100% level. However, the humidity level did not decrease as much as desired, and at the maximum temperature, relative humidity was 85% rather than 62%. Similar observations were made in the basic hot condition, with one exception. Due to severe discomfort reported by the subjects, maximum temperature was 41°C rather than 44°C, and maximum was not maintained for a full hour. The problems with humidity control probably resulted from excessive ambient humidity during this time period. The capacity of the main environmental control system in the building was exceeded. Overall, the environmental conditions achieved were somewhat more severe than expected.

As expected, efficacy was lower in conditions which caused excessive perspiration. In the hot/humid and variable hot conditions, SL-30 formulation had significantly lower repellency and shorter CPT than HSL-50 (Table 14). Concurrently, it was observed that SL-30 had a greater Troop Acceptability Score than HSL-50. Therefore, a formulation with the cosmetic elegance of SL-30 and the repellency of HSL-50 was sought during reformulation. The result was HSL-44. In the basic hot condition, this formulation showed slightly, but not significantly, greater efficacy, measured as mean percent repellency or CPT, compared to HSL-50. As discussed above, no differences were observed under ambient conditions.

TABLE 13

MOSQUITO EFFICACY TESTS

CLIMATE CONDITIONS

TIME (HOUR)	TEST SESSION HOT/HUMID		TEST SESSION VAR/HUMID		TEST SESSION BASIC HOT	
	TEMPERATURE (°C)	HUMIDITY REL. %	TEMPERATURE (°C)	HUMIDITY REL. %	TEMPERATURE (°C)	HUMIDITY REL. %
0	28	83	26	72	92	60
1	32	94	26	94	30	60
2	33	82	27	97	31	74
3	32	88	32	92	33	72
4	34	80	38	95	35	72
5	41	70	35	85	36	71
6	41	62	33	92	41	62
7	38	65	33	87	34	56
8	32	73	30	92	36	61
9	32	71	29	93	33	62
10	32	74	34	98	33	70
11	32	81	28	93	29	61
12	32	90	29	89	29	80

Table 14

REPELLENT EFFICACY*

ADVERSE CONDITIONS

TIME (HOURS)	HSL-44 BASIC HOT	HSL-50 HOT/HUMID	HSL-50 VAR. HOT	HSL-50 BASIC HOT	SL-30 HOT/HUMID	SL-30 VAR.HOT
2	100.0	100.0	100.0	100.0	100.0	100.0
4	100.0	100.0	100.0	100.0	100.0	97.1±2.9
6	100.0	85.5±12.6	97.1±2.9	100.0	56.2±18.2	84.1±8.1
8	97.6±1.5	80.0±20.0	100.0	98.7±1.3	2.3±2.3	71.3±19.7
10	75.0±11.2	54.6±18.8	.4±18.0	81.4±5.1	7.1±7.1	25.2±14.7
12	56.4±18.0	14.8±8.0	31.0±14.5	62.7±15.2	-	0.00
CPT	10.0±0.9	9.6±1.0	9.2±0.8	10.0±0.6	6.0±0.0	7.2±1.2
n	5	5	5	5	5	5

* Data are presented as mean percent repellency ± S.E.M.
**CAM indicates efficacy when repellent was applied over camoflague face paint.
***CPT = Complete protection time

D. Troop Acceptability

1. Methodology

Troop acceptability tests were conducted by H. V. Shuster, Inc., as a subcontract to BIOTEK. The method was developed by the subcontractor based on their standard methods of sensory testing and ASTM STP 434. Subjects were naive adults of military age, 18 to 36 years old. Subjects were seated two to a table in a specially designed consumer testing laboratory. Informed consent was obtained from each subject, and the test procedure explained. Participants were instructed to roll their sleeves up to the elbow and rub a sample on the inside of their forearm. The order of presentation was randomized. Without further instruction, subjects were asked to fill out a questionnaire. The protocol, informed consent form, and questionnaire are presented in Appendix II, the final report submitted by H. V. Shuster, Inc.

2. Experiments and Results

Three repellent formulations were tested for troop acceptability. In the first test, HSL-50 and SL-30 were evaluated by 12 male and 13 female subjects. The males ranged from 23 to 36 years old with a mean of 28 ± 4 (S.E.M.) years, and females were between 28 and 36 years old with a mean of 31 ± (S.E.M.) years. The raw data and data summaries are presented in Tables VII to IX of Appendix II. The mean hedonic scale score for SL-30 was 5.3 and for HSL-50; 4.0 which corresponds to "Like Slightly" and "Neither Like Nor Dislike" respectively. For SL-30, 72% of the respondents rated the material as "Like Slightly" or higher and 92% rated it as "Neither Like Nor Dislike". The corresponding values for HSL-50 were 48% and 68% respectively. Concurrent efficacy studies (see Section C demonstrated that HSL-50 was more efficacious in high humidity conditions than SL-30. Based on these data, the sustained action repellent was reformulated with the objective of improving both efficacy in high humidity and cosmetic elegance. The resulting formulation, HSL-44, was rated as "Like Slightly" or higher by 72%, and rated as "Neither Like Nor Dislike" or higher by 84% of a panel consisting of 13 men and 12 women. The women on this panel ranged from 22 to 33 years old with a mean of 28 ± 3 (S.E.M.) years, and the men were between 23 and 36 years old with a mean of 28 ± 4 (S.E.M.) years. The mean score for HSL-44 was 5.1 out of a possible 7.

Based on these data, both HSL-44 and SL-30 marginally meet the specifications for troop acceptability. A major difference was noted when the data was analyzed on the basis of sex of the panelists. For both of these products, 85% of the male panelists gave ratings of "Like Slightly" or better. One possibility is that females who use cosmetics regularly are more attuned to cosmetic elegance. However, female panelists used were not naive. They had participated in similar evaluations of other cosmetic products, and this experience may have introduced a bias.

The major objection to these materials was their high viscosity and a sticky or tacky feel. These characteristics were most prominent with HSL-50 and most noticeable during application. After application, formulation HSL-44 and SL-30 had mean scores of "Good" or better when panelists rated "Feel of Product on the Skin". At least 80% of the subjects rated the odor of the product as moderate or less. It was interesting that 44% of the panelists stated on the Comments question that HSL-44 had an agreeable odor.

E. Toxicology

1. Methodology

Toxicology tests required for E.P.A. registration of the sustained release arthropod repellent were conducted in compliance with Good Laboratory Practices rules and regulations (40 CFR 160). Testing was carried out in BIOTEK's fully AAALAC accredited animal facility following Standard Operating Procedures. All work complies with AAALAC standards as set forth by the "Guide for the Care and Use of Laboratory Animals", of the Institute of Laboratory Animal Resources, National Resource Institute, DHEW Publication (NIH) 78-23, 1978. We acknowledge that the conduct and reporting of the studies described adhered to the principles outlined in the above guide.

a. Primary Eye Irritation

Eye irritation was evaluated using female New Zealand White rabbits obtained from Pine Acres Rabbitry, West Brattleboro, VT. The rabbit is a universally accepted, classical model for study of eye irritation. Animals were quarantined and observed for at least seven days prior to study. Only healthy animals were selected for study.

The study animals andomly selected for testing had their eyes examined at least 24 hours prior to compound administration using fluorescein dye procedures. Only those animals with no sign of corneal injury or eye abnormalities were utilized. One eye of each animal was used as the test eye, the contralateral eye serving as the untreated control for that animal. Animals used were divided into two groups, Group I consisting of six rabbits and Group II consisting of three rabbits.

Test material (0.1 ml) was dropped onto the corneal surface above the averted lower lid of the rabbits' eye. The upper and lower lids were then gently held together for ten seconds before releasing to prevent loss of material. Group II animals had the treated and control eyes flushed for one minute with lukewarm water within 20 to 30 seconds after compound instillation. The eyes of the rabbits in Group I remained unwashed.

The treated eyes of both groups were read for ocular lesions at 1, 24, 48, and 72 hours, and at 7 days after treatment. If injury was present at 7 days, observations continued at 14 days. At the 72-hour and 7-day readings and all subsequent readings, sodium fluorescein and a cobalt blue light was used to aid in revealing possible corneal injury. Grading and scoring of irritation was performed using the Draize technique (Draize, 1959). All eye abnormalities observed were recorded.

b. Primary Dermal Irritation

Dermal irritation was evaluated using female New Zealand White rabbits obtained from Pine Acres Rabbitry, West Brattleboro, VT. The rabbit is a universally accepted, classical model for evaluating skin irritation. Animals were quarantined and observed for at least seven days prior to the study. Only healthy animals were selected for study.

Twenty-four hours prior to test material application, the hair was clipped from the back and flanks of six randomly selected female rabbits. Abraded areas were made on three exposed areas of each rabbit to provide three abraded and three intact test sites. The sites on the left side were abraded for three rabbits and the right side was abraded for the remaining three rabbits. The abrasions were deep enough to penetrate the stratum corneum but not deep enough to penetrate to the dermal layer and cause bleeding.

Each test material was applied to two areas on each rabbit, one abraded area and one intact area, in the amount of 0.5 ml per area. The treated areas were each covered with 2.5 x 2.5 cm gauze patch (two single layers thick), secured with tape, and overwrapped with Saran Wrap™ and tape to maintain the test material in contact with the skin and decrease the rate of evaporation.

After the 24 hours of exposure, the patches were removed and the test material was removed as thoroughly as possible without irritating the skin. Thirty minutes following compound removal, the degree of erythema and edema was recorded according to the Draize technique (Draize, 1959). A second reading was taken at 72 hours to determine the primary irritation index for the sample. If irritation was seen at the 72-hour examination period, observations were repeated at 96 hours, and also at 7 and 14 days, if dermal irritation persisted.

c. Dermal Sensitization

Dermal sensitization was evaluated in male Hartley guinea pigs, approximately 500 grams at the beginning of the study, using the closed patch technique. Animals were obtained from Charles River Breeding Laboratories, Wilmington, MA and quarantined and observed for at least 7 days prior to the study. The guinea pig is a universally accepted, classical model for sensitization studies. Only healthy animals were selected. Assignment of animals to study groups and selection of application sites was done by randomization. Ten animals were selected for each of 2 test groups, and four animals were selected for positive control.

Twenty-four hours prior to each application, the hair was removed from the back of each animal with electric clippers. Test material was applied to one area on each animal by placing the appropriate amount of test material on a gauze pad (7/8" x 1") and placing the pad on the test site along the midline of the back. The patch was covered with Saran Wrap™ and secured with an overwrap of tape. The dressing remained in place for a period of six hours at which time it was removed.

The animals received three applications per week (Monday, Wednesday, Friday) for three weeks for a total of nine applications. Each application was alternated between the right and left sides along the midline of the animal and location (right or left, anterior or posterior) of the initial test site was determined by randomization. Dinitrochlorobenzene was used as the positive control at a concentration of 0.3% in 50% ethanol. The study method used for the control groups was identical to that of the test group.

Two weeks following the administration of the ninth sensitizing dose, a challenge dose was administered to all groups in the same manner as during the sensitizing phase of the study. Approximately 24 hours prior to application of

the challenge dose, the hair was removed from the back of each animal with electric clippers. The test or control material was applied to a test site used during the sensitization phase of the study and to a second, previously untreated test site. Forty-eight hours after the first challenge, a second challenge was made in the same manner as described above.

The application sites were read and scored for erythema and edema at 7, 24 and 48 hours following each application according to the Draize technique (Draize, 1959). Reactions to the challenge doses were read and scored at 7, 24, and 48 hours as was done following sensitizing applications.

On the third day of the sensitization phase, formulation SL-30 was discarded due to poor efficacy at high humidity. At this point, HSL-44 was substituted for SL-30.

2. Experiments and Results

The toxicology tests required by the FDA for an end use product containing formulated Deet prepared from registered technical grade Deet are: Primary Dermal Irritation, Dermal Sensitization, and Primary Eye Irritation. These tests were conducted using sustained release formulation developed by BIOTEK. The methods are presented in Section III.I.

a. Primary Dermal Irritation

Twenty-four hours following the application of 500 ul of SL-30, HSL-44, and HSL-50 to abraded and intact rabbit skin, mild, barely preceptible erythema was observed with all formulations. Slight edema was observed 72 hours after application of HSL-44 to the skin. The data is summarized in Table 15. Slight but statistically insignificant irritation was observed with each formulation. The Primary Irritation Index (Draize, 1959) ranged from 0.54 ± 0.10 (S.D.) for SL-30 to 0.92 ± 0.66 for HSL-44. It may be significant that HSL-44 appears somewhat more toxic than HSL-50 which contains a higher Deet/matrix concentration.

These results are very similar to those reported in the "N,N-diethyl-m-toluamide (Deet) Registration Standard", Office of Pesticides and Toxic Substances, Environmental Protection Agency, Washington, DC (1980). The results of six studies referenced in the standard for formulated Deet show no irritation at 72 hours. Two studies using technical grade Deet demonstrated slight edema and erythema 48 hours following application and no irritation at 7 days. The current study and the referenced studies indicated that BIOTEK sustained action arthropod repellent is in Toxicity Category IV corresponding to a very low primary dermal irritation potential.

b. Skin Sensitization

Throughout the nine applications of test material, only slight irritation was observed. The four positive controls, however, showed perceptible erythema during the sensitizing treatments. Following a two-week drug-free period, the animals were challenged twice. Challenge with either HSL-44 or HSL-50 gave no observable dermal response except for one animal in each group. The chlorodinitrobenzene positive control, however, showed a response on the first challenge significantly greater than the first sensitizing treatment. Similar

Table 15

SUSTAINED ACTION ARTHROPOD REPELLENT

PRIMARY DERMAL IRRITATION[1]

Formulation	Initial Body Weight (kg)	Primary[2] Irritation Index	24-Hour Observation				72-Hour Observation					96-Hour Observation			
			Abraded		Unabraded		Abraded		Unabraded		Body Weight (kg)	Abraded		Unabraded	
			Er[3]	Ed[4]	Er	Ed	Er	Ed	Er	Ed		Er	Ed	Er	Ed
HSL-44	2.96 ± 0.36	0.9 ± 0.7	1 ± 0	0 ± 0	1 ± 0	0 ± 0	0.5 ± 0.8	0.3 ± 0.5	0.5 ± 0.8	0.3 ± 0.5	3.04 ± 0.36	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0 ± 0
HSL-50	-	0.6 ± 0.2	1 ± 0	0 ± 0	1 ± 0	0 ± 0	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0 ± 0	-	0.2 ± 0.4	0 ± 0	0.2 ± 0.4	0 ± 0
SL-30	-	0.5 ± 0.1	1 ± 0	0 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.4	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[1] Based on the method of Draize (1959). Data are presented as means ± S.D.
[2] See Draize (1959)
[3] Erythema Score - See Draize (1959)
[4] Edema Score - See Draize (1959)

results were observed following the second cnallenge. The data are summarized in Tables 16, 17, and 18.

The data indicate that BIOTEK's sustained action Deet formulations are not sensitizers. The positive control indicates that the study was conducted in a proper manner. Overall results are very similar to those reported in the "N,N-diethyl-m-toluamide (Deet) Registration Standard", Office of Pesticides and Toxic Substances, Environmental Protection Agency, Washington, DC (1980).

c. Primary Eye Irritation

The three sustained action formulations demonstrated significant eye irritation. The data is summarized in Table 19. In this study, there was a slight corneal opacity which only occasionally obscured details of the iris. However, the opacity observed generally involved at least half of the corneal surface. At the 24-hour observation and subsequent observations, 2% opthalmic fluorescein was instilled in both control and treated eyes, and the eyes observed with a cobalt blue penlight (Concept, Inc., Clearwater, FL).

The fluorescein examination generally confirmed the observations of corneal damage. In all cases where general corneal damage was reported, there was a slight but obvious staining of the cornea. It was easier to discern the area affected using fluoroscein and the cobalt blue light.

For each of two formulations, HSL-50 and HSL-44, the eyes of three rabbits were washed. By the seventh day, the washed eyes containing HSL-50 were normal while the unwashed eyes were still near peak irritation. For HSL-44, washing did reduce the level of irritation but only 1 of the 3 rabbits was normal. It should be noted that a minimal degree of corneal opacity covering the cornea is equal to a score of 20.

These results were unexpected. Pilot studies conducted on a prototype formulation containing 20% Deet showed that all irritation had cleared by day seven without washing. The results of the pilot studies appeared very similar to those cited in the "Deet Standard". It was also surprising that HSL-50, which contains a higher concentration of Deet/matrix than HSL-44, caused less severe eye irritation with washing.

These data indicate that the BIOTEK prototype formulation causes more prominent eye irritation than technical grade Deet and should be regarded as a severe eye irritant. Based on the results of pilot studies and the relative toxicity observed for HSL-44 and HSL-50, this study should be repeated. While the effects of Deet concentration and the ratio of Deet in the matrix are not clear, the observed results are difficult to interpret. If the toxicity is due to a sustained release of toxic material at the local site, then one would predict the irritation should be proportional to Deet concentration. It was not. Further studies should also include Deet controls so that a direct, rather than indirect, comparison can be made.

Table 16

SUSTAINED RELEASE ARTHROPOD REPELLENT HSL-50

DERMAL SENSITIZATION IN GUINEA PIGS[1]

Sensitizing Treatment Number	7-Hour Observation		24-Hour Observation		24-Hour Observation	
	Er[2]	Ed[3]	Er	Ed	Er	Ed
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0.1±0.3	0	0	0	0	0
Challenge Treatment						
1	0	0	0	0	0	0
2	0	0	0	0	0	0

[1] Data are presented as means ± S.D. (except where S.D. = 0). Dermal irritation scoring is by the method of Draize (1959). n = 10

[2] Er = Erythema

[3] Ed = Edema

Table 17

SUSTAINED RELEASE ARTHROPOD REPELLENT

1,CHLORO-2,4 DINITROBENZENE

DERMAL SENSITIZATION IN GUINEA PIGS[1]

POSITIVE CONTROL

Sensitizing Treatment Number	7-Hour Observation		24-Hour Observation		24-Hour Observation	
	Er[2]	Ed[3]	Er	Ed	Er	Ed
1	1	0	0	0	0	0
2	1	0	1	0	1	0
3	1	0	0.2±0.5	0	0	0
4	1	0	1	0	1.5±0.6	0
5	1	0	1	0	1	0
6	1	0	1.0±0.8	0	1.0±0.8	0
7	1	0	1.2±0.5	0	1.2±0.5	0
8	1.5±0.6	0	1.5±0.6	0	1.5±0.6	0
9	1.8±0.5	0	1.5±0.6	0	1.2±1.0	0
Challenge Treatment						
1	1.8±0.5	0	1.8±0.5	0	1.8±0.5	0
2		0		0		0

[1] Data are presented as means ± S.D. (except where S.D. = 0). Dermal irritation scoring is by the method of Draize (1959). n = 4

[2] Er = Erythema

[3] Ed = Edema

Table 18

SUSTAINED RELEASE ARTHROPOD REPELLENT HSL-44
DERMAL SENSITIZATION IN GUINEA PIGS[1]

Sensitizing Treatment Number	7-Hour Observation		24-Hour Observation		24-Hour Observation	
	Er[2]	Ed[3]	Er	Ed	Er	Ed
1*	0	0	0	0	0	0
2*	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
Challenge Treatment						
1	0	0	0	0	0.1±0.3	0
2	0	0	0	0	0	0

[1] Data are presented as means ± S.D. (except where S.D. = 0). Dermal irritation scoring is by the method of Draize (1959). n = 10

[2] Er = Erythema

[3] Ed = Edema

Another prototype formulation, SL-30, was administered in the first two treatments. Efficacy tests indicated that this was not satisfactory so the formulation was altered to HSL-44, and the experiment continued.

Table 19

SUSTAINED ACTION ARTHROPOD REPELLENT
PRIMARY EYE IRRITATION[1]

Formulation	MEAN TOTAL DRAIZE SCORE UNWASHED (± S.D.) 1 Hour	24 Hour	48 Hour	72 Hour	7 Days	14 Days	Body Weight (kg) Initial	Final
HSL-44	26.5±6.1	41.0±1.8	42.5±9.4	42.5±11.5	31.2±18.9	15.5±11.8	3.3±0.20	3.25±0.18
HSL-50	24.0±10.0	40.7±14.6	34.3±23.4	34.0±15.1	32.8±29.1	8.5±8.1	3.12±0.17	3.14±0.17
HSL-30	17.5±2.8	35.8±12.8	32.7±16.9	39.5±21.9	27.0±13.9	19.7±18.2	3.15±0.19	3.16±0.21

Formulation	MEAN TOTAL DRAIZE SCORE WASHED 1 Hour	24 Hour	48 Hour	72 Hour	7 Days		Body Weight (kg) Initial	Final
HSL-44	15.7±2.9	24.3±18.8	12.7±11.6	17.0±17.3	10.7±14.4	1.7±2.9	3.40±0.24	3.30±0.22
HSL-50	14.7±1.2	19.7±15.3	5.7±4.7	4.3±5.9	0.0±0.0	0.0±0.0	3.28±0.16	3.30±0.16
SL-30	—	—	—	—	—	---	—	—

[1] Based on the scoring system of Draize (1959)
Maximum Draize Score = 110

F. Military Material Compatibility Tests

1. Methodology

In order to determine the effect of our sustained release Deet formulation on military materials, a series of test protocols, modeled after the ASTM methods outlined in Symposium on Toxic Control* (1981) were developed. The following materials were selected for evaluation after preliminary consultation with the project officer. The protocols for each test procedure are listed in Appendix III.

Table 20 lists the test procedure numbers, the type of materials tested, and their possible applications.

2. Experiments and Discussion

The current in use Army Deet formula, the alcoholic lotion base, and BIO-TEK's sustained release Deet formulation (HSL-44) were all tested. The raw data is listed in Appendix IV. The compatibility of the three formulations with each class of materials is discussed below.

a. Plastics

1. Cellulose acetate butyrate was totally solvated by the current Army preparation and by the sustained release Deet formulations. It was not affected by the alcoholic lotion base at all.

2. Low density polyethylene was not affected by any of the three formulations. The slight gloss loss produced by the alcoholic lotion base was due to the presence of a thin residue of silica after vacuum drying.

3. Methyl methacyrlate suffered a slight gloss loss after being subjected to the current Army Deet formulation. There were no visible changes observed when it was subjected to the lotion base or sustained release formula.

4. Nylon 6/6 was not visibly affected by any of the three test formulations.

5. Polyacrylamide samples were affected by all test formulations. Although there were no significant weight or dimensional changes in each sample, a definite loss of gloss was observed. A greyish film also formed on the surface of each sample.

6. Polycarbonate samples were affected by both the current Army repellent and the sustained release Deet formulation. Both formulations produced a slight cloudiness. The alcoholic lotion base had no visible effect.

7. Polyurethane samples were also affected by both the Army and sustained release Deet formulations. Samples A and C showed evidence of swelling and a definite tackiness was observed. The current Army Deet formulation also discolored the sample, whereas the sustained release formulation did not. The polyurethane sample was not visibly affected by the alcoholic lotion base.

*Symposium on Toxic Control: Decontamination, June 1981, U. S. Department of Commerce, National Technical Information Service.

Table 20

Materials Testing Protocols

Test Procedure Number	Materials	Application
	1) Plastics	
P-1	a) Cellulose Acetate Butyrate	Eyeglass Nosepiece
P-1	b) Low Density Polyethylene	General Equipment
P-1	c) Methyl Methacrylate (Plexiglass)	Safety Glass
P-1	d) Nylon 6/6	Clothing
P-1	e) Polyacrylamide (Lucite)	Facemasks
P-1	f) Polycarbonate (Lexan)	Lenses
P-1	g) Polyurethane	Rifle Stocks
	2) Adhesives	
AD-1	a) Surgical Tape	Medical & Surgery
AD-2	b) Adhesive Bandages	Use
	3) Rubber and Elastomers	
RE-1	a) Latex Rubber	Surgical Gloves
RE-1	b) Silicone Rubber	Tires
	4) Organic Coatings	
O-1	a) Auto Enamel	Automotive Paint
O-2	b) Acrylic Camouflage Paint	Camouflage
O-1	c) Polyurethane	Rifle Stocks
	5) Metals	
M-1	a) Aluminum	General Equipment
	6) Natural Products	
N-1	a) Leather	Boots
	7) Textiles	
T-1	a) Cotton (100%)	Miscellaneous Clothing
T-1	b) Cellulose Acetate (Rayon)	Miscellaneous Clothing
T-1	c) Cotton-Nylon (50/50)	Battle Dress Uniform
T-1	d) Polyester (100%)	Miscellaneous Clothing

b. Adhesives

1. The sustained release Deet formulation had less of a detrimental effect on surgical tape than the current Army Deet formulation when compared to an untreated specimen.

2. Adhesive bandages which were subjected to the alcoholic lotion base showed a 2% increase in adhesive strength when compared to the current Army Deet formulation.

Adhesive bandages which were subjected to the sustained release Deet formulation showed a 4.4% decrease in adhesive strength when compared to the current Army Deet formulation.

c. Rubber and Elastomers

1. All three samples of latex rubber were unaffected by any of the test formulations.

2. All three samples of silicone rubber were unaffected by any of the test formulations.

d. Organic Coatings

1. Automobile enamel was unaffected by the current Army Deet formulation, the alcoholic lotion base, and the sustained release formula. The scratch and gouge test results indicate a hardness of 4H which remained the same after treatment in all three cases.

2. The current Army Deet formulation and the sustained release formulation were virtually identical in their effect on the acrylic camouflage paint. Both formulations removed approximately 42% of effective camouflage. The alcoholic lotion base removed virtually all effective camouflage due to its high viscosity.

3. Polyurethane coated samples were attacked by the current Army Deet formulation more so than the sustained release formulation. There was visual evidence of deterioration, bubbling, and solubility. The gouge and scratch tests resulted in a rating of less than 6B. The sustained release Deet formulation was less detrimental and was given a rating of 5B for gouge hardness and a rating of 4B for scratch hardness, although there was a definite loss of gloss. The alcoholic lotion base also attacked the polyurethane finish but to a lesser extent as both the gouge and scratch hardness ratings were 5B. The area was slightly tacky to touch and a definite loss of gloss was noted.

e. Metals

Aluminum was not visibly affected by all three solutions.

f. Natural Products

Leather was not visibly affected by all three solutions.

g. Textiles

Of the four textiles tested, only the 50/50 cotton-nylon B.D.U. was affected. A leaching of dark green dye was noted when samples of the 50/50 cotton-nylon B.D.U. were immersed in both the current Army Deet formulation and the sustained release Deet formulation. The alcoholic lotion base had no effect on any of the textiles. There was no fiber damage or change in weave when all the samples were viewed under a 7X stereomicroscope.

Overall, the effect of BIOTEK's sustained release Deet formulation on military materials is similar to the current in-use Army Deet formualtion.

G. Manufacturing of Sustained Release Repellent

1. Formulation

Alcohol lotion base 3002-42B was prepared according to the formula developed in conjunction with H. Y. Shuster of Quincy, MA (see Appendix II). The lot used in our final repellent formulation, to be delivered at the conclusion of Phase I, was prepared as shown in Table 21.

Ingredients were mixed in a heated stirring vessel until homogenous The HSL-44 controlled release formulation was then prepared using the above lotion base, BIOTEK's Deet/silica blend, and SDA-40. The final formulation is shown in Table 22.

2. Package Design

While a unique packaging system was desired, we were limited at this stage by the great expense involved in producing a prototype container in such small quantities (110 samples). The minimum charge for a custom color in a production line bottle is $5,000 and the minimum charge for developing and producing a custom bottle is about $40,000.

The prototype repellent package supplied is a 2 oz., white, low density polyethylene drug oval bottle with a 3.0 mm Polytop™ dispensing closure. The Polytop™ closure (Figure 34) provides ease in dispensing, and in contrast to the current in-use package, there is no cap to be unscrewed and lost. The closure is leak proof and can be operated with one hand in contrast to those dispensing caps which require a verticle pull to open. Polytop™ closures are sturdier than most 1 piece, snap-top dispensing closures. This closure will be used in the final package.

BIOTEK proposes that our final package will be a 2 oz. low density polyethylene bottle, shaped significantly different from the current in-use bottle, colored dark green, brown, or multi-color camouflage (molded-in or silk-screened on). The closure will be a dark green, brown, or camouflage 3.0 mm Polytop™ closure. Labeling will be directly silk screened on the container with repellent resistant ink. The selection of bottle style and color will be based on a consumer panel evaluationg of several packages compared to the current Army container (see Figure 34).

Table 21

Alcohol Lotion Base 3002-42-B

Component	Generic Name	Weight (%)	Weight (g)
Lipocol C Lot #P-227F4 Lipo Chemicals, Inc. Paterson, New Jersey	Cetyl Alcohol NF Grade	25.0	457.4
Hydrofol Acid 16555NF Lot #5051-M639-531 Sherex Chemical Co., Inc.	Stearic Acid NF Grade	3.5	64.0
Trolamine Lot #M120 Olin Corporation Stamford, Connecticut	Triethanolamine 99%	2.0	36.6
Solulan 98 Lot #508 Amerchol Edison, New Jersey	Polysorbate 80 Cetyl Acetate Acetylated Lanolin Alcohol	41.5	759.3
SDA 40 Lot # 8-19-85 Aaper Alcohol, Inc. Shelbyville, Kentucky	Specially Denatured Alcohol-40 200 Proof	28.0	512.3
TOTAL		100.0	1829.6 g

Table 22

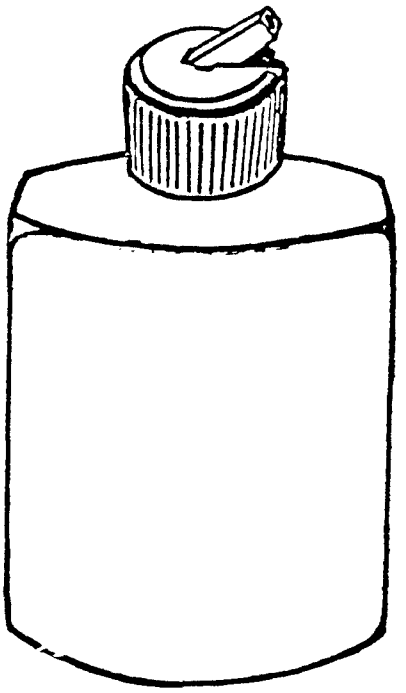
BIOTEK Sustained Action Arthropod Repellent Formula

Component	Weight (%)	Weight (g)
BIOTEK Deet/Silica Blend	58.7	4800
Alcohol Lotion Base 3002-42-B	14.7	1200
SDA-40 Alcohol	26.7	2182
TOTAL	100.0	8182 g

These components were blended thoroughly and the final formulation dispensed into 2.0 oz. low density polyethylene bottles with Poly-top™ closures.

Figure 34

BIOTEK Prototype Repellent Package



2 oz. classic oblong bottle (LDPE) with Poly-Too™ closure.

H. Conclusion

BIOTEK has developed a sustained-release personal use arthropod repellent containing N,N-diethyl-m-toluamide (Deet). This repellent formulation, when applied to exposed skin at an average dose of 2 mg/cm^2, will provide protection for a duration of twelve hours or more. This has been achieved by formulating Deet with a proprietary blend of silica gel, alcohol, emollients and suspending agents. The formulation is non-toxic, is cosmetically acceptable, and is compatible with most military materials.

I. Recommendations

The effect of Hydrophobic Silica Gel (HSG)-Deet weight ratios and concentration of the HSG-Deet matrix in our final formulation have not been studied completely. Efficacy, toxicity, cosmetic acceptability and military material compatibility may all be improved by further refinement.

More formulation development of emollients and suspending agents has been done, but due to time constraints, these have not been tested. These new formulations promise improved cosmetic acceptability with no detrimental effect on efficacy.

The effect of making silica gel Type B hydrophobic should also be studied. The greater pore volume of Type B silica, when hydrophobic, may provide greater efficacy. Water repellency of Type B matrices may also be improved by this process.

All future repellent development and screening should utilize the in vitro methods developed in Phase I of this project, but primary emphasis should be placed on human studies, i.e. in vivo and field studies, as well as troop acceptability studies.

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V. APPENDICES

Appendix I	Materials and Supplies
Appendix II	Herbert V. Shuster, Inc. Report No. 92505
Appendix III	Military Materials Compatibility Testing Protocols
Appendix IV	Military Materials Compatibility Testing Results

APPENDIX I

MATERIALS AND SUPPLIES

Equipment	Model/Type	Supplier	Use
Gas Chromatograph	Perkin-Elmer Sigma 2B w/Sigma 10B Data Station	Perkin-Elmer Norwalk, CT	Deet Analysis
Gas Chromatograph Column	18" x 1/8" SS, 0.01 SP-1000 on 80/100 Carbopack C	Supelco, Incorporated Bellefonte, PA	Deet Analysis
Penetration/Evaporation Apparatus	Skin Permeation Systems Model LG-1083-C	Laboratory Glass Apparatus Berkeley, CA	In Vitro
Silica Gel	Tell-Tale	Davison Chemical Baltimore, MD	In Vitro
Condenser	5979-12	Ace Glass, Incorporated Vineland, NJ	In Vitro
Deet Absorbant	Tenax GC 60/80	Alltech Associates Arlington Heights, IL	In Vitro
Air Peristaltic Pump	Masterflex Multichannel w/ (3) 7014 Heads	Cole Palmer Instrument Co. Chicago, IL	In Vitro
Flowmeters	R-3216-04 0-50 cc/min Std. Air	Cole Palmer Instrument Co. Chicago, IL	In Vitro
In-Line Bubblers	7532-10	Ace Glass Incorporated Vineland, NJ	In Vitro
Ringer's Lactate Pump	Ismatek MV-MS-3 3 Channel	Cole Palmer Instrument Co. Chicago, IL	In Vitro
Penetration Cell Fluid	Lactated Ringer's	Abbott Laboratories N. Chicago, IL	In Vitro
Water Bath/Circulator	Masterline 2095 Bath and Circulator	Forma Scientific Marietta, OH	In Vitro
Scintillation Cocktail	Biofluor	New England Nuclear Boston, MA	In Vitro
Scintillation Counter	Beckman LS-1000	Beckman Instruments, Inc. Irvine, CA	In Vitro
Air Vacuum Pump	Air Cadet	Cole Palmer Instrument Co. Chicago, IL	In Vitro

MATERIALS AND SUPPLIES (Cont).

Equipment	Model/Type	Supplier	Use
Liquid Peristaltic Pump	Masterflex Drive w/ 10 Channel Head	Cole Palmer Instrument Co. Chicago, IL	In Vitro
Flowmeters	100-1000 cc/min Std. Air	Dwyer Instruments, Inc. Michigan City, IN	In Vitro
Micro Syringes	Micropettors Models A, E, & C	Scientific Manufacturing Emeryville, CA	In Vitro
Tissue Solubilizer	Protosol	New England Nuclear Boston, MA	In Vitro
Tissue Solubilizer	Biogest	Fisher Scientific Springfield, NJ	In Vitro
Hairless Mice Substrate	Male	Buckshire Laboratories Perkasie, PA	In Vitro
Pigskin Substrate	Yorkshire (Weanling)	Tufts School of Veterinary Medicine North Grafton, MA	In Vitro
Shaver	Remington Micro Screen	Remington Products, Inc. Bridgeport, CT	In Vitro
Dermatome	Padgett Model B	Padgett Dermatome Kansas City, MO	In Vitro
Humidity Meter	Airguide	Airguide Instrument Co. Chicago, IL	In Vivo
Micro Syringes	Micropettors Models A, E, & C	Scientific Manufacturing Emeryville, CA	In Vivo
Psychrometer	Bacharach Sling	Bacharach Pittsburg, PA	In Vivo
Textiles	Battle Dress Uniform	Mass. Army Navy Boston, MA	Compatibility
Silicon Rubber	Silastic Grade NRV	Dow Corning Corp. Midland, MI	Compatibility
Magnifier	Optivisor	W.C.O. Randolph, MA	Toxicology

APPENDIX II

Report Prepared for Biotek, Inc.

by: Herbert V. Shuster, Inc.

Report No.: 92505
Work Order No.: 26324
Date: September 4, 1985

Respectfully submitted,

HERBERT V. SHUSTER, INC.

Kurt S. Konigsbacher, D.Sc.
Vice President, Corporate Development

To: Biotek, Inc.

Attn: Dr. E. S. Nuwayser

Report No.: 92505

Date: September 4, 1985

SUMMARY

Aqueous emulsion cream bases and alcoholic bases were formulated to be converted into arthropod repellents with a minimum of 30 percent and up to a maximum of 60 percent microencapsulated DEET. It was found that the stability of a repellent with an aqueous emulsion cream base was not sufficient for a sustained release product because of the incompatibility of even microencapsulated DEET with water.

Emphasis was placed on the development of alcoholic bases and several products were formulated that were cosmetically elegant and had satisfactory stability after microencapsulated DEET was added.

The best formulations were tested in vivo for 12 hour periods under ambient and very severe environmental conditions. The sustained release repellents remained effective over the 12 hour period at ambient environmental conditions. Under severe temperature and humidity conditions, the test formulations remained completely effective for eight to ten hours and retained partial efficacy over the whole twelve-hour test period.

The two best repellents were acceptable to 72 percent of a small population group (25 people). Men only judged the repellents acceptable 85 percent of the time. Both figures are believed to be statistically significant. Mean acceptance scores for the repellents were between "Like Slightly" and "Like Moderately." Both, feel on the skin and odor level were generally judged to be in acceptable ranges. The major complaint was a tendency for the repellent to be somewhat sticky, which was found to be a factor of DEET.

To: Biotek, Inc.

Attn: Dr. E. S. Nuwayser

Report No.: 92505

Date: September 4, 1985

OBJECTIVE

The objective was to develop an aqueous emulsion cream and an alcoholic base compatible with microencapsulated DEET. When mixed with the microencapsulated arthropod repellent DEET (N, N-diethyl-m-toluamide), the formulation was designed to provide a protection time of up to twelve hours at the 95 percent confidence level.

In addition, the finished formula had to have troop acceptance (improved odor and feel), be compatible with fabric and face paint, and perform over the total test period at extreme environmental conditions.

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

Page 3

BACKGROUND

The current mosquito repellent used by the Armed Forces has low acceptance and consequently is not used by troops because of its objectionable odor and feel. The reason for the objectionable odor and feel is the high concentration of active ingredient (75%) necessary to provide even minimal protection for 2.5 hours.

Since arthropods are repelled by odor, effectiveness depends on the rate at which the repellent is released into the air. The ability to penetrate the skin also limits the effectiveness time provided by DEET. The partition coefficient serves as a measure for organic molecules to pass through lipoproteins such as the skin. The optimal partition coefficient for dermal penetration, 1.0, is representative of substances such as DEET which are soluble in both polar and non-polar solvents.

It was our endeavor to formulate prototype bases which, when mixed with DEET modified by microencapsulation, would lead to improved effectiveness time. This meant that bases and finished repellents had to be developed in which the DEET remained in the microencapsulated state as long as required to give the desired rate of release over a 12 hour test period.

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

EXPERIMENTAL PROCEDURES AND RESULTS

1. Formulation of Repellent Bases

Taking into account the nature of the products involved, the circumstances under which they are to be used and the fact that the container has to fit into a shirt pocket, the concept of a pump spray or other advanced delivery system was abandoned for the time being. Instead, the bases and finished products were designed to go into basic oval polyethylene or polypropylene squeeze bottles with a typical closure that would not leak in the closed position.

Theoretical considerations indicated that the minimum ratio of microencapsulated DEET in the formula to be released over a 12 hour period was 30 percent. The bases were, therefore, formulated for a 30 percent minimum DEET concentration. However, since there was a good chance that higher concentrations of DEET may have to be used to remain effective under extreme environmental conditions, base formulations were designed that could accommodate higher concentrations of DEET without becoming cosmetically unacceptable.

a) Aqueous Emulsion Cream Bases

Aqueous emulsion cream bases were formulated specifically to be compatible with microencapsulated DEET. The goal was to develop a cosmetically acceptable product that was stable in the presence of microencapsulated DEET. It was found that a satisfactory repellent could be formulated with 30 percent of microencapsulated DEET, particularly the DEET identified as HS.

Of the many different types of formulas developed, as shown in Table I formula 2968-19A was found to be the most suitable. Its composition is given in Table II

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

EXPERIMENTAL PROCEDURES AND RESULTS (Con't)

On further in vivo evaluation of the sustained release characteristcs of an aqueous emulsion cream repellent, it was found that the water, even in the form of an emulsion, decreased the stability of the microencapsulated DEET to the point where it was no longer effective over a sustained period of time. Therefore, the utilization of an aqueous emulsion cream base was judged to present a poor risk/value ratio and emphasis was placed on the development of non-aqueous bases.

b) Alcoholic Bases

Several series of alcoholic bases were formulated to be compatible with microencapsulated DEET. The goal was to develop cosmetically acceptable bases that could tolerate up to 50 percent of their weight in microencapsulated DEET. To be useful to the Armed Forces, the repellent had to be sufficiently viscous, not to drip or make camouflage paint run, yet it had to be thin enough to be squeezed readily from the container and be spread on the skin without discomfort.

Of the many different types of formulas developed, as shown in Table III product 3002-42B was found to be most stable in the presence of microencapsulated DEET, identified as HS, and products 3002-42B (modified) and 3147-17E in the presence of microencapsulated DEET, identified as HSG. The formulas and their laboratory method of preparation are given in Tables IV through VI .

It became evident during the development of alcoholic bases that the DEET strongly affected the physical properties of the bases and that the changes were roughly proportional to the quantity of DEET added. With increasing quantities of microencapsulated DEET, the repellent became more viscous until it was essentially a gel. It also became more sticky,

EXPERIMENTAL PROCEDURES AND RESULTS (Con't)

which made it more difficult to apply and cosmetically less acceptable. However, the most successful formulas developed to date were still satisfactory when 40 to 50 percent of microencapsulated DEET was added.

2. Efficacy and Acceptance Testing of Repellent

a) Implementation of FDA REgulations

To be able to carry out a clinical test program with patients, it was necessary to go through the initial steps required by the Food and Drug Administration.

- A specific and detailed protocol was designed to determine the efficacy of sustained release arthropod repellents. The procedure is shown as Figure 1
- A subject informed consent form was prepared for the sustained release mosquito repellent. A copy of the form is shown as Figure 2
- The protocol, the subject informed consent form, background information on the toxicity of DEET itself and other pertinent information was submitted to the acting chairman of the SHUSTER Institutional Review Board for an expedited review.
- The test program was approved by the expedited IRB review procedure with the proviso that each patient be informed at the time of signing the informed consent form that there was a slight possibility the product might irritate the skin.
- A second protocol and subject informed consent form were designed and submitted for an expedited Institutional Review Board approval for the troop acceptance study. The test program was also approved using the expedited IRB review procedure. The protocol and the Informed Consent Forms used are shown as Figures 3 and 4

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

EXPERIMENTAL PROCEDURES AND RESULTS (Con't)

b) Efficacy Testing

The sustained release arthropod repellents described as S/L/30 (30% DEET), HS/L/44 (44% DEET) and HS/L/50 (50% DEET) were tested for their efficacy using the protocol shown as Figure 1. The results of the tests are described in Tables VII through IX.

The data showed that the sustained release repellents remained effective over the whole 12 hour test period at normal environmental conditions. Under hot and humid conditions, product S/L/30 remained effective for 6 hours and partially effective for 8 hours. Product HS/L/50 retained its efficacy for 8 hours and remained partially effective over the whole 12 hour test period. Under basic hot environmental conditions, both product HS/L/44 and HS/L/50 remained completely effective for 8 hours and retained partial efficacy for the whole 12 hour test period. This particular test was actually unduly severe and flawed because the virgin mosquitoes used were of poor quality and had a tendency to die in the cage before the cage was applied to the skin. Thus, the number of live mosquitoes per cage averaged just under 15 (from 6.4 to 18.8) instead of the usual 20.

c) Acceptance Testing

The arthropod repellents were also tested for troop acceptance in a separate test program where a small quanity of each product was applied by the participants to the skin of their forearms. The products were then rated on a seven-point hedonic scale and for feel and odor level on five-point rating scales. The scale for feel ranged from excellent (5) to poor (1), while the scale for odor level ranged from very strong (5) to none (1). The participants were also asked to provide the reasons for the scores given. The actual score sheet used is shown as Figure 5.

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

EXPERIMENTAL PROCEDURES AND RESULTS (Con't)

The results of the tests are summarized in Tables X through XII.

The data indicated that the formulas that contained less encapsulated DEET were liked better because they felt better on the skin and were cosmetically more elegant because they were less sticky. The results from testing the latest alcohol-based repellent were most encouraging because they showed almost the 75 percent acceptance established by the Armed Forces as the lower permitted limit. On the basis of a population group of 25, acceptance was 72 percent (18/25, 19/25 = 76%) for products S/L/30 and HS/L/44. The actual mean values for the two repellents were 5.3 and 5.1 respectively; i.e., both values were above the "like slightly" category on the hedonic scale. It is interesting to note that the acceptance figure for male participants only was 85 percent. Apparently, women who use cosmetics regularly are much more attuned to cosmetic elegance.

The feel on the skin ranged from fair for repellant HS/L/50 to good for HS/L/44 and good to very good for product S/L/30. The odor level was generally judged to be between slight and moderate with practically no difference between the three repellents.

To: Biotek, Inc. Report No.: 92505

Attn: Dr. E. S. Nuwayser Date: September 4, 1985

CONCLUSIONS

From the repellent base formulation program, it was concluded that an elegant and stable aqueous emulsion cream could be developed with a satisfactory release of the active component. However, the incompatibility of DEET with water, even when microencapsulated, limited the concentration of DEET that could be added to the base and of the controlled release time that could be achieved.

Emphasis was placed, therefore, on the development of a cosmetically elegant alcohol-based product. Several formulations were found to provide good release characteristics and to be compatible with different ratios of microencapsulated DEET. At least one formula tested showed satisfactory troop acceptance with 44 percent DEET added.

From the development program, it was also concluded that the repellent loses cosmetic elegance and becomes more sticky as the concentration of microencapsulated DEET reaches 60 percent.

From the efficacy tests, it was concluded that the best repellents were entirely satisfactory over the 12 hour period under ambient conditions. Under severe environmental conditions, the best formulations remained entirely satisfactory up to 10 hours and were reasonably effective over the whole 12 hour test period.

Based on the base development studies to date and the acceptance and efficacy tests conducted, it was concluded that it should be possible to formulate an even more elegant and efficient alcohol-based base and that a repellent can be designed that retains its efficacy for at least 12 hours under the most severe environmental conditions without being affected by camouflage paint.

The following Tables I through XIII and Figures 1 through 5 are a part of Report No. 92505.

TABLE I

AQUEOUS EMULSION BASES PREPARED FOR BIOTEK PROJECT

	Emulsifier Type	Emulsifier(s)	Auxillary Stabilizer*	Formula#
1.	Anionic	Stearate Soap	-	3002-20A
2.	Nonionic	Polysorbate & Alkanolamide	-	3002-20B
3.	Nonionic	Polyglycol Stearate	-	2968-15A
4.	Nonionic	Polypropylene Glycol Cetyl Phosphate	-	2968-15B
5.	Nonionic/Anionic	Polyglycol Stearate & Stearate Soap I	-	3002-21A
6.	Nonionic/Anionic	Nonoxynol Phosphate & Polyglycol Stearate & Stearate Soap	-	3002-21B
7.	Nonionic/Anionic	Polygylcol Stearate & Stearate Soap II	-	2968-16A
8.	Nonionic/ Cationic	Polygylcol Lanolin Alcohol & Stearamine I	-	2968-17A
9.	Nonionic/ Cationic	Polygylcol Lanolin Alcohol & Stearamine II	-	2968-21B
10.	Nonionic/ Cationic	Polyglycol Soya Alcohol & Diethylamino Stearate I	-	2968-17B
11.	Nonionic/ Cationic	Polyglycol Soya Alcohol & Diethylamino Stearate II	-	3002-27B
12.	Nonionic/ Cationic	Lapyrium Chloride & Glyceryl Stearate & Amine Oxide	-	3002-22
13.	Nonionic/Anionic	Polyglycol Laurate & Stearate Soap	+	2968-18A
14.	Nonionic	Polyglycol Palmitate	+	2968-18B
15.	Nonionic	Polyglycol Stearate I	+	2968-21A
16.	Nonionic	Polyglycol Stearate II	+	3002-27A
17.	Nonionic	Polyglycol Stearate III	+	3002-28
18.	Anionic	Stearate Soap I	+	2968-19A
19.	Anionic	Stearate Soap II	+	3002-23
20.	Anionic	Stearate Soap III	+	3002-24A
21.	Anionic	Stearate Soap & Nonoxynol Phosphate	+	3002-24B

* Auxillary Stabilizer is Carbomer 934, a crosslinked polyacrylic acid.

TABLE II

INSECT REPELLENT BASE #2968-19A

CTFA/UPS/NF Designation	Vendor Name	% w/w
Part A		
Cholesterol NF	Croda	0.5
Cetyl Alcohol NF	Sherex Adol 52 NF	0.5
Glyceryl Stearate CTFA/CID	Kessco Stepan	1.0
Cetyl Palmitate CTFA/CID	Armak Kessco 653	1.0
Octyldodecanol CTFA/CID	Henkel Standamul	3.5
Stearic Acid NF	Emery Emersol 132	1.5
Part B		
Carbomer 941 (1% suspension) CTFA/CID	BF Goodrich Carbopol 1941	10.0
DIH_2O		81.4
Part C		
Triethanolamine 99% CTFA/CID	Olin	0.6
		100.0

Procedure

Heat Part A & B separately to 75°C. Add Part B to Part A and mix, maintaining temperature, for 10 minutes to effect emulsion. Add Part C. Cool, with stirring, to 30°C.

Insect Repellent Lotion

	Wt. %
Base 2968-19A	71.0
DEET Preparation HS	29.0
	100.0

Procedure

Mix at room temperature with propellar agitation until homogenous.

TABLE III

ANHYDROUS ALCOHOL FORMS PREPARED FOR BIOTEK PROJECT

DEET Source	Dispersant/Emollient Agents		Solvent/Diluent
(Prep.HS)	Solulan 98	Other(s)	(SD Alcohol 40,Anhydrous
50	10	Crodamol PTC 10	30
50	15	Crodamol PTC 15	20
50	20	-	30
50	-	Crodamol PTC 20	30
50	30	-	20
50	-	Crodamol PTC 30	20
50	-	Acetylated Lanolin Alcohol 20	30
50	-	Cetyl Alcohol 20	30
30	35	-	35
30	40	-	30
30	45	-	25
60	25	-	15
55	20	Cetyl Alcohol 10	15
50	20	Cetyl Alcohol 15	15
40	25	Cetyl Alcohol 15	20
60	-	Polysorbate 20 25	15
60	-	Steareth 100 25	15
60	-	Steareth 20 25	15
60	-	PEG 50 Stearate 25	15
60	-	PEG 16 Lanolin Alcohol 25	15
50	20	Cetyl Alcohol 15/TEA Stearate 2.5	12.5
40	25	Cetyl Alcohol 15/TEA Stearate 3.2	16.8
55	-	Cetyl Alcohol 10/Polysorbate 20 20	15
55	-	Cetyl Alcohol 10/Polysorbate 20 25	10
60	-	Cetyl Alcohol 10/Polysorbate 20 20	10
55	20	Cetyl Alcohol 15/TEA Stearate 1.5	8.4
55	25	Cetyl Alcohol 10/TEA Stearate 1.5	8.4

Notes:- All figures given are % by weight.
- Solulan 98 is the name of a proprietary mixture consisting of Polysorbate 80, Cetyl Acetate and Acetylated Lanolin Alcohol.
- Crodamol PTC is Croda, Inc., name for Pentaerythritol Tetra Caprate/Caprylate.
- TEA is an abbreviation for triethanolamine.

The remaining ingredients are more fully described in the USP/NF, Merck Index or CTFA Cosmetic Ingredients Dictionary.

TABLE III

ANHYDROUS ALCOHOL FORMS PREPARED FOR BIOTEK PROJECT

DEET Source	Dispersant/Emollient Agents		Solvent/Diluent
	Solulan 98	Other(s)	(SD Alcohol 40,Anhydrous)
50 (S-2)	20	Cetyl Alcohol 15/ TEA Stearate 25	12.5
40 (S-2)	25	Cetyl Alcohol 15/ TEA Stearate 3.2	16.8
67 (HSG)	8	Cetyl Alcohol 5	20
60 (HSG)	8	Cetyl Alcohol 5	27
55 (HSG)	9	Cetyl Alcohol 5.5	30.5
67 (HSG)	7.5	Cetyl Alcohol 4.5/TEA Stearate 1.0	20
60 (HSG)	7.5	Cetyl Alcohol 4.5/TEA Stearate 1.0	27
54 (HSG)	7.5	Cetyl Alcohol 4.5/TEA Stearate 1.0	33
60 (HSG)	6.2	Cetyl Alcohol 3.75/TEA Stearate 0.85	29.2

TABLE IV

3002-42B Base (With DEET HS)

Ingredient	% w/w	Specification	Approx. Cost/lb.
Cetyl Alcohol	25.0	NF	$ 1.13
Stearic Acid	3.5	NF	0.53
Trolamine	2.0	NF	0.63
Polysorbate 80 (and) Cetyl Acetate (and) Acetylated Lanolin Alcohol	41.5	Solulan 98 (Amerchol)	2.66
Specially Denatured Alcohol #40, Anhydrous	28.0	USP and 27CFR Part 212	0.50
	100.0		$ 1.55/lb of base

Laboratory Preparation:

Ingredients are added together into a suitable mixing vessel and mixed until dissolved and homogeneous. Mild heat is necessary to attain complete dissolution. Q.S. batch to appropriate weight with alcohol to compensate for any loss on heating. Base will solidify on standing and should be warmed to liquefy it before use. Test formulation is prepared by mixing DEET HS and warmed base until mixture is uniform.

This base was prepared for use with Biotek sample DEET HS. The initial sample submitted for testing was a mixture consisting of 40% DEET HS (21% DEET) and 60% Base. To increase the DEET content of the product, as much as 55% DEET HS (28.5% DEET) can be blended with 45% Base before tackiness and stickiness become apparent and the application properties are affected.

TABLE V

Modified 3002-42B Base (With DEET HSG)

Ingredient	% w/w	Specification	Approx. Cost/lb
3002-42B Base	37.8	(see above)	$ 1.55
Specially Denatured Alcohol #40, Anhydrous	62.3	(see above)	0.50
	100.0		$ 0.90/lb of base

55% of DEET HSG (41% DEET) was blended with 45% of Modified 3002-42B Base to form a product with excellent rub-in characteristics with little trace of stickiness/tackiness. This is a cosmetically superior product yet contains 41% DEET. If the ratio of DEET HSG is increased to 60% and above, application properties are altered.

TABLE VI

3147-17E Base (With DEET HSG)

Ingredient	% w/w	Specification	Approx. Cost/lb.
Cetyl Alcohol	12.5	NF	$ 1.13
Polysorbate 80 (and) Cetyl Acetate (and) Acetylated Lanolin Alcohol	20.0	Solulan 98 (Amerchol)	2.66
Specially Denatured Alcohol #40, Anhydrous	67.5	USP and 27CFR 212	0.50
	100.0		$ 1.01/lb

60% of DEET HSG (45% DEET) can be blended with 40% of 3147-17E Base and still apply as a very cosmetically acceptable product.

Summary:

DEET is a sticky/tacky liquid and a maximum of 40-45% of DEET can be incorporated into alcoholic cosmetic bases before its properties dominate the system.

* Cam = Camouflage Paint on Skin
** Number of bites

TABLE VII (20 Mosquitoes/Cage)

EFFICACY AT AMBIENT TEMPERATURE

Product	Patient	Number of Bites												
		2 Hours		4 Hours		6 Hours		8 Hours		10 Hours		12 Hours		
		Cam*	Nothing	Cam	Nothing	Cam	Nothing	Cam	Nothing	Cam	Nothing	Cam	Nothing	Control
S/L/30	DC	-	0**	-	0	-	0	-	0	-	0	-	0	15
	JM	-	0	-	0	-	0	-	0	-	0	-	0	9
	MD	-	0	-	0	-	0	-	0	-	0	-	0	4
	RH	-	0	-	0	-	0	-	0	-	0	-	0	10
	RD	-	0	-	0	-	0	-	0	-	0	-	0	13
	Av.	-	0	-	0	-	0	-	0	-	0	-	0	
HS/L/50	DC	-	0	-	0	-	0	-	0	-	0	-	0	
	JM	-	0	-	0	-	0	-	0	-	1	-	0	
	MD	-	0	-	0	-	0	-	0	-	1	-	0	
	RH	-	0	-	0	-	0	-	0	-	0	-	0	
	RD	-	0	-	0	-	0	-	0	-	0	-	0	
	Av.	-	0	-	0	-	0	-	0	-	0.4	-	0	10.2
HS/L/50	JM	0	0	0	0	0	0	0	0	0	0	0	0	20
	CC	0	0	0	0	0	0	0	0	0	0	0	0	13
	TP	0	0	0	0	0	0	0	0	0	0	0	0	15
	JD	0	0	0	0	0	0	0	0	0	0	0	0	20
	DD	0	0	0	0	0	0	0	0	0	0	0	0	14
	Av.	0	0	0	0	0	0	0	0	0	0	0	0	16.4
HS/L/44	JM	0	0	0	0	0	0	0	0	0	0	2	0	13
	CC	0	0	0	0	0	0	0	0	0	0	0	0	13
	TP	0	0	0	0	0	0	0	0	0	0	0	0	19
	JD	0	0	0	0	0	0	0	0	0	0	0	0	16
	DD	0	0	0	0	0	0	0	0	0	0	0	0	10
	Av.	0	0	0	0	0	0	0	0	0	0	0.4	0	14.2

[redacted]

* White Powder Appeared on Skin and Remained Through Test

(20 Mosquitoes/Cage)

• Skin Reddened

•• Initial: T:31°C, R.H.: 88%; Maximum: T:41°C, R.H.: 52%

•• Initial: T:26°C, R.H.: 100%; Maximum: T:35°C, R.H.: 62%

TABLE VIII

EFFICACY UNDER HOT AND HUMID CONDITIONS

Product	Patient	Number of Bites						
		2 Hours	4 Hours	6 Hours	8 Hours	10 Hours	12 Hours	Control
S/L/30••	DC	0	0*	10	11	13	-	10
	DA	0	0	4	15	16	-	10
	DB	0	0*	16	14	19	-	10
	MD	0	0*	1	15	16	-	16
	JD	0	0	2	16	8		16
	Av.	0	0	6.6	14.2	14.4	-	12.4
S/L/30••	CB	0	2	3*	6	12*	19	14
	KA	0	0	0	0	9	17*	14
	JM	0	0	2	0*	18	16	6
	JOM	0	0	0	0*	3	19	15
	JD	0	0	6	15	15	19	20
	Av.	0	0.4	2.2	4.2	11.4	18.0	13.8
HS/L/50••	DC	0	0*	0	0	2	11	
	DA	0	0	0	0	8	10	
	DB	0	0*	8	15	16	15	
	MD	0	0**	0	0	7	13	
	JD	0	0*	1	0	0	7	
	Av.	0	0	1.8	3	6.6	11.2	
HS/L/50••	CB	0	0*	0	0	4*	4	
	KA	0	0	0	0	11	17*	
	JM	0	0	2*	0	18	10	
	JOM	0	0	0	0*	2	6	
	JD	0	0	0	0	15	18	
	Av.	0	0	0.4	0	10.0	11.0	

[redacted]

* White Powder Appeared on Skin and Remained Through Test

° Skin Reddened

°° Initial: T:30°C, R.H.: 44%; Maximum: T:43°C, R.H.: 22%

°° Expressed as Percent Instead of Average Because of Poor Mosquitoes, Some of Which were Dead in Cage as Cage was Applied to Arm.

(20 Mosquitoes/Cage)

TABLE IX

EFFICACY UNDER BASIC HOT CONDITIONS

Product	Patient	Number of Bites						
		2 Hours	4 Hours	6 Hours	8 Hours	10 Hours	12 Hours	Control
HS/L/44°°	JM	0°	0	0°	1	6	5	17
	JOM	0	0	0°	1	1	3	10
	DB	0	0	0°	0	1	0	14
	RH	0	0	0°	0	2	1	17
	JM	0°	0	0	0	0	4	19
	Percent°°	0	0	0	2.2	22	40.5	83
HS/L/50°°	JM	0°	0	0°	1	2	1	
	JOM	0	0	0	0°	2	2	
	DB	0	0	0°	0	1	3	
	RH	0	0	0°	0	2	2	
	JM	0°	0	0	0	0	12	
	Percent°°	0	0	0	1.1	14	34	

* For Scale See Figure 5

TABLE X

ACCEPTANCE OF MOSQUITO REPELLENTS

Participant	Score S/L/30	HS/L/50	HS/L/44
A	4*	3	7
B	5	4	6
C	6	5	5
D	6	6	7
E	6	1	6
F	6	4	6
G	6	1	7
H	6	6	6
I	5	4	2
J	5	6	3
K	6	5	5
L	3	5	5
M	7	6	7
N	2	5	4
O	4	1	4
P	4	4	3
Q	5	3	6
R	5	2	6
S	6	5	4
T	7	3	6
U	6	4	5
V	7	6	3
W	4	2	5
X	4	5	5
Y	7	5	5
Av. Score	5.3	4.0	5.1

TABLE XI

REASONS FOR HEDONIC SCORES

Participant	Product S/L/30	HS/L/50	HS/L/44
	Less Tacky (3)*	Too Thick (6)	Nice Odor (11)
	Sticky (2)	Too Sticky (10)	Not too Sticky (5)
	Less Odor (4)	Oily (2)	Sticky (5)
	Sandpaper (1)	Good Odor (1)	Spreads well (1)
		Strong Odor (2)	Doesn't Spread Well (1)

* Number of times mentioned.

* For Scale See Figure 5 (Appendix)

TABLE XII

SUMMARY OF FEEL ON SKIN

Participant	Feel on Skin S/L/30	HS/L/50	HS/L/44
A	2*	1	5
B	3	2	5
C	3	2	5
D	4	3	4
E	3	1	4
F	3	2	4
G	2	1	4
H	4	3	3
I	3	2	4
J	3	5	3
K	3	2	3
L	2	3	3
M	4	3	3
N	4	1	?
O	3	1	2
P	3	3	1
Q	2	2	2
R	3	1	2
S	3	3	3
T	4	1	4
U	4	2	3
V	4	3	1
W	5	2	2
X	3	3	2
Y	5	2	2
Av. Score	3.3	2.2	3.0

TABLE XIII

SUMMARY OF ODOR LEVEL

Participant	Odor Level S/L/30	HS/L/50	HS/L/44
A	3	3	3
B	2	3	3
C	2	3	2
D	2	2	2
E	2	2	3
F	3	4	2
G	4	3	2
H	2	3	3
I	3	3	4
J	3	2	4
K	3	3	3
L	2	2	3
M	2	4	2
N	5	2	2
O	3	3	5
P	3	2	2
Q	2	2	3
R	3	3	3
S	3	2	2
T	2	3	4
U	1	2	4
V	4	2	2
W	2	3	2
X	2	3	3
Y	4	4	2
Av. Score	2.7	2.7	2.8

Figure 1
EXHIBIT C

BIOTEK

SUSTAINED RELEASED ARTHROPOD REPELLENT

Efficacy Protocol

OBJECTIVE:

1. Demonstrate that a sustained release formulation of N,N-diethyl-m-toluamide will reduce the number of bites by female Aedes aegypti mosquitoes by 95% compared to untreated control for at least 12 hours.

2. Demonstrate that concurrent use of camouflage face paint does not interfere with the efficacy of the sustained release formulation.

3. Determine the effect of modified climatic conditions on the efficacy of the sustained release formulation.

SUMMARY OF METHOD

A constant dose of sustained release arthropod repellent is applied to the ventral surface of subjects forearms. The test sites are protected from abrasion throughout the experimental period. Each test site is repeatedly exposed to mosquitoes, contained in test chambers, in a defined testing environment. Each subject will serve as his own control. Since this is not a comparison test, repellency is quantitated as percent of bites on treated site compared to the untreated control site.

PROCEDURE:

1. The efficacy of a sustained action arthropod repellent will be tested on male subjects of military age (18-36 years) who have not shown a sensitivity reaction to insect bites and have given their informed consent.

2. The subjects forearms will be washed with a natural soap which does not contain perfume, deodorants, or other cosmetic enhancers and rinsed with 70% ethanol on the morning of the experiment.

3. Control and Test Sites:

   a. For each subject, a control site on either the proximal or distal end of either the right or left ventral forearm will be selected by a random number table previously generated. A 5 cm diameter circle will be marked with a waterproof pen to define the site. Care must be taken to insure that no repellent is placed on this site.

   b. For each subject a test site 5 to 7 cm by 15 to 20 cm will be marked on each ventral forearm with a waterproof pen. The dimensions of the test site will depend on the size of the subjects forearm. The area of each test site will be determined for dose calculation.

NOTE 1 - The test and control sites must not overlap. If the subject has a small forearm the test site may be smaller.

4. Test materials will be applied at a maximum dose of 30 mg of deet per sq. cm. to the test sites and the time recorded. The volume or weight of test material to give the rquired dose is calculated from the area of the test site. It is placed on the skin, and spread evenly over the test site with a finger or blunt glass rod.

   NOTE - 2 This maximum dose is approximately 10 times the cummulative dose of deet required with repeated applications for 95% repellency for 10 to 24 hours. The actual dose will be determined in pilot studies.

5. Repellency will be tested at 2 hour intervals for 6 tests (i.e. 2, 4, 6, 8, 10, and 12 hours following application). For each subject, one test interval, selected by a random number table previously generated, will include tests of both test sites and the control site. For the other test intervals only test sites will be tested.

6. For each test, subjects will be comfortably seated with forearms supported on a table, in the testing laboratory which will be maintained between 20 and 25°C and approximately 50% relative humidity and constant light.

7. Test chambers containing a specific number (between 20 and 50) female Aedes aegypti mosquitoes will be secured with Velcro™ straps over the appropriate test sites on the forearms 1 minute before beginning the test.

   NOTE 3 - The specific number of mosquitoes used will be determined in pilot studies.

8. At test time the protective sliding closure will be removed from the test chamber to expose the test sites to mosquitoes.

9. For each test the test sites will be exposed to mosquitoes for a specified period of time between 1.5 and 3 minutes. At the completion of the test the protective sliding closure will be replaced, the test chambers will be removed from the forearms and the mosquitoes destroyed.

   NOTE 4 - The specified duration of the test session will be determined in pilot studies. The time should be sufficient for 95-100% of the mosquitoes to bite an untreated control site.

10. During each test the following observations shall be recorded: (1) the number of mosquitoes that land but depart without probing; (2) the number that probe but depart without feeding; and (3) the number that feed to repletion or are interrupted during feeding by termination of the test. Either probing or feeding will define a bite.

11. Between tests the subjects may engage in normal activities. However, test subjects must avoid any contact with the treated skin surfaces. Touching, rubbing or abrasive action on the treated skin can affect the results. Subjects should wear short sleeve shirts. Avoid undue sweating or wetting of the treated skin except in special tests designed to study the durability of repellents under adverse conditions. Loss due to evaporation and absorption is, of course, unavoidable but should be the only known reason for loss in these tests, if the above precautions are taken.

SPECIFIC EXPERIMENTAL CONDITIONS:

Efficacy tests are required for the following conditions. For each subject assignment of treatment to each test site will be based on a random number table previously generated.

NOTE 5 - For untreated control sites a large number of bites will occur. For the comfort of the subjects these bites will be treated either by rinsing with hot water or a commercial insect bite product. Since it is not known how previous bites in an area effect subsequent biting behavior, an area which has received more than one bite will not be retested. In that case the test chamber will be placed over another location on the test site.

1. The repellent formulation alone and in combination with camouflage face paint.

   a. For this condition 10 subjects will be required.
   b. Test and control sites will be prepared as described in the procedure (Section 4).
   c. Camouflage face paint will be applied to one test site, selected by a random number table previously generated, following the instructions on the label.
   d. Sustained release arthropod repellent will then be applied to both test sites as described in the procedure (Section 4).
   e. Testing will be conducted as described in the procedure.

2. Climatic Design Test

   a. Three conditions which are modifications of the Climate Design Types described in AR 70-38 have been selected. Maximum temperature and humidity changes are compressed into the 12 hour test period. Temperature will be increased slowly such that maximum temperature will be reached at the end of the fifth hour of the experiment. Maximum termperature will be maintained for one hour and then slowly decreased to reach the initial temperature at the beginning of the eleventh hour.

Figure 1 (Con't)

Condition	Initial Temperature	Initial Relative Humidity	Maximum Temperature	Maximum Relative Humidity
Hot-humid	31°C	88%	41°C	52%
Variable high/ humidity	26°C	100%	35°C	62%
Basic-hot	30°C	44%	43°C	22%

b. Approximately five subjects will be assigned to each Climatic Design Type.

c. Following application of sustained release arthropod repellent to the test site subjects will be exposed to the climate condition. At test time the subjects will return to the testing laboratory for efficacy testing. This is necessary since variation in temperature and humidity effects mosquito feeding behavior. Following efficacy testing subjects will return to the assigned climatic condition until the next test.

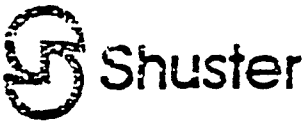
DEFINITIONS

1. Deet - N,N-diethyl-m-toluamide.
2. 95% Protection level - The number of bites on a test site is not more than 5% of the number of bites observed on the same subject's control site.
3. Landing - Mosquito touches the skin but does not probe or feed.
4. Probing - Mosquito penetrates the skin, repeatedly moves the mouth parts, and the papilla vibrate.
5. Feeding - Mosquito stops moving the mouth parts and the papilla are still.
6. Repletion - Following feeding, a mosquito leaves the site before termination of the test.
7. Bite - A bite is either a probing or feeding response.

MATERIALS AND SUPPLIES

1. Testing Laboratory - An area maintained at 20-25°C and approximately 50% relative humidity, lighting should be even and constant.
2. Instruments to determine temperature, humidity and light levels.
3. Clock (1) and stopwatches (1 for each observer).
4. Mosquito test chambers.
5. Aedes aegypti female mosquitoes in transport cages.
6. Apparatus to transfer mosquitoes from transport cage to test chambers and from test chambers to disposal chambers.
7. Data sheets and pens.
8. Natural soap for washing and 70% ethanol in water for rinsing skin.
9. Commercial insect bite treatment (O.T.C.).
10. Comfortable seating at a table for subject and observer during testing.
11. Waterproof pens for marking skin.
12. Random number table for assigning subjects and test sites to treatment conditions.
13. A list of all chemicals to be used with pertinent toxicological data.
14. Environmental chambers for climatic design conditions.

Figure 2



BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

I, _____(Full Name, Please Print), fully understand that Herbert V. Shuster, Inc., is engaged in a research study with Biotek, Inc., to develop and evaluate the effectiveness of a sustained release mosquito repellent formulated with DEET (N, N-diethyl-M-toluamide) as the active ingredient and to be applied as an alcoholic liquid and/or as an emulsified lotion. The product is being developed for the Armed Forces under the sponsorship of the United States Army Medical Research and Development Command. The repellent is to be used on men of military age (18-36 years) who are not sensitive to insect bites.

I understand that DEET has been widely used by the Armed Forces in mosquito repellents without untoward effects and that this active ingredient is of low toxicity and is not known to be a potential skin irritant or sensitizer. I further understand that the repellent will be applied to the forearm(s). A plastic test cage with a predetermined number of mosquitoes will be placed on the forearm and held in place with Velcro straps. A protective slide will be removed and the mosquitoes will have access to 3.1 square inches of skin for 1.5 to 3 minutes. The protective slide will then be closed, and the test cage removed. The test site will be exposed to the mosquitoes at two-hour intervals over a twelve-hour period. A control site will be exposed only once. The test design also will evaluate concurrently whether the use of camouflage face paint interferes with the efficacy of the sustained release formulation and whether modified climate conditions (temperatures ranging from 20 to 43°C and relative humidity ranging from 22 to 100%) affect the efficacy. The mosquitos used are laboratory-bred virgin mosquitoes free of disease and that have not been experimentally used previously. Total duration of the experiment will be 14 hours.

I hereby acknowledge that the following points concerning this study have been thoroughly explained to me.

1. Dr. Elie S. Nuwayser, Biotek, Inc., 21-C Olympia Avenue, Woburn, MA, 01801, (617) 938-0938 is the Principal Investigator in this study who may be contacted.

2. I have been advised that there will be some discomfort and irritation from the bites, which will be treated as quickly as possible.

3. Accidental exposure of the repellent product to the eyes or mucous membranes should be avoided because the product can be an irritant to those areas.

Herbert V. Shuster Inc
Quincy Research Park
5 Hayward Street
Quincy, Massachusetts 02171
Telephone 617 328-7600

BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

4. I have been given the opportunity to ask all questions regarding the study, and they have been answered to my satisfaction.

5. I do understand that I must keep my scheduled appointments, since this is important for the success of the study. My failure to do so will result in my disqualification as a test subject.

6. I will follow the specific directions given to me and will not willfully do anything that might change the results of the study.

7. If I have any questions during the study, I should contact Dr. Kurt S. Konigsbacher, Vice President, Corporate Development at (617) 328-7600.

8. If I am injured as a direct result of administration of the test materials, I understand that medical treatment shall be made available through Herbert V. Shuster, Inc. Financial compensation for such things as lost wages, disability or discomfort due to this type of injury is not available. I understand, however, that I have not waived any of my legal rights by signing this form.

9. I understand that participation in this study is voluntary and I may withdraw from the study at any time. If I am dismissed from the study for medical reasons (such as inability to tolerate the test products or procedures), my compensation will be proportional to the time spent in the study. If I am dismissed from the study because I have not complied with the protocol or withdraw from the study without valid reason, I shall receive no compensation at all.

As a volunteer participating in the study outlined above, I acknowledge and certify that:

1. I am aware that the United States Army Medical Research and Development Command is sponsoring the study.

2. I have been questioned as to my knowledge of present and past allergies. I understand that I should not participate in the study if I have any allergies or skin sensitivity to insect bites or skin preparation products.

3. I am aware of the nature, purpose and duration of the study and that the foreseeable health risks of any participation in the study are limited to the adverse reactions of the products being tested. In addition, there may be other risks which are unforeseeable.

BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

4. I am aware that I will not directly receive health benefits from the study.

5. I am aware that copies of all data obtained during the course of this study are the property of the sponsor. My name will be disclosed to the sponsor. I am also aware that if this study is ever subject to inspection by the Food and Drug Administration or other government agencies, the government investigators may review the data as well as obtain copies of it and/or this informed consent. I understand that all this information is considered by Herbert V. Shuster, Inc., as strictly confidential.

6. I am aware that any questions I might have concerning the procedures which affect me will be answered promptly and completely to my satisfactior by the Principal Investigator or by Dr. Kurt S. Konigsbacher.

7. I am aware that I have the right to withdraw my consent and discontinue my participation in this study at any time.

8. I am aware that this study involves a total of ten (10) subjects for efficacy evaluation of the formulation alone and in combination with the camouflage face paint and fifteen (15) subjects for the climate design test.

9. I have the legal capacity to sign this informed consent.

10. I am aware that I will receive a copy of this informed consent.

Executed this _____ (day) day of _____ (month), 1985.

Subject Name: _____

Address: _____

City: _____, Zip _____

Phone No.: _____

Birthdate: _____

Identity Verification: _____

Soc. Sec. No.: _____

Witnessed by: _____ (Staff Member)

LABORATORY METHODS MANUAL	HVS, Inc. Test Method No.:	HS-II-L
HERBERT V. SHUSTER, INC. 5 HAYWARD STREET NORTH QUINCY, MASSACHUSETTS	Date:	7/10/85
	Supersedes:	None
	Page: 1 of 2	

1. Title: Study to Determine Acceptability of Mosquito Repellent
2. Method: Sensory
3. Reference: Manual on Sensory Testing Methods ASTM STP 434
4. Validation: N/A
5. Application: Mosquito Repellent

6. Principle:
   - 6.1 Mosquito repellent is applied to human skin and evaluated by human volunteers for acceptability.
7. Materials and Reagents:
   - 7.1 Pre-measured 1 gram samples of mosquito repellent.
8. Apparatus:
   - 8.1 N/A
9. Procedure:
   - 9.1 Standard Preparation:
     - 9.1.1 None
   - 9.2 Sample Preparation:
     - 9.2.1 None
   - 9.3 Test Procedure:
     - 9.3.1 Before beginning testing:
       - 9.3.1.1 Documentation that the samples pass animal testing for dermal toxicity must be available before the products are used on human subjects.
       - 9.3.1.2 Documentation of the protocol approval by our expedited Institutional Review Board (IRB) must be available before the testing begins.

Prepared By: Katharine Agnes
Date: 7/10/85

Approved By: [illegible]
Date: 7/11/85

LABORATORY METHODS MANUAL HERBERT V. SHUSTER, INC. 5 HAYWARD STREET NORTH QUINCY, MASSACHUSETTS	HVS, Inc. Test Method No.: HS-II-L Date: 7/10/85 Supersedes: None Page: 2 of 2

Title: Study to Determine Acceptability of Mosquito Repellent

9. Procedure: (Cont'd)

9.3 Test Procedure: (Cont'd)

9.3.2 Subjects:

9.3.2.1 A minimum of twenty-five subjects are chosen randomly from a naive adult population 18 to 36 years old.

9.3.3 Time Frame:

9.3.3.1 The material is evaluated immediately after application.

9.3.4 Procedure:

9.3.4.1 Subjects are seated two to a table in our consumer testing area at a specified time. Talking is prohibited.

9.3.4.2 Subjects are requested to complete a subject informed consent form. (Figure 1)

9.3.4.3 The testing is explained to each panelist. They are then given a questionnaire to read. (Figure 2)

9.3.4.4 The panelist is then asked to roll their sleeves up to the elbow.

9.3.4.5 Participants are given the sample and instructions to rub the sample on the inside of their forearm. The order of presentation (if there is more than one sample) is randomized to prevent position bias.

9.3.4.6 Without further instructions, participants are asked to complete the questionnaire.(Figure 2)

10. Calculation:

10.1 None

11. Precision:

11.1 N/A

12. Reporting Results:

12.1 The samples are reported as "Pass" or "Fail". If seventy-five percent of the participants rate the material "like slightly", "like moderately" or "like very much", the material, by definition, passes the acceptability test.

13. Special Precautions:

13.1 None

Prepared By: Katherine Ayers Approved By:

Date: 7/10/85 Date:

Figure 4

Shuster

BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

I, _____(Full Name, Please Print), fully understand that Herbert V. Shuster, Inc., is engaged in a research study with Biotek, Inc., to develop and evaluate the effectiveness of a sustained release mosquito repellent formulated with DEET (N, N-diethyl-M-toluamide) as the active ingredient and to be applied as an alcoholic liquid and/or as an emulsified lotion. The product is being developed for the Armed Forces under the sponsorship of the United States Army Medical Research and Development Command. The repellent is to be used on healthy individuals of military age (18-36 years) who are not sensitive to insect bites. Pregnant or lactating women will be excluded.

I understand that DEET has been widely used by the Armed Forces in mosquito repellents without untoward effects and that this active ingredient is of low toxicity and is not known to be a potential skin irritant or sensitizer. I further understand that the repellent will be applied to the forearm of each volunteer who will then be asked how well he likes the new mosquito repellent. The method of choice will be a seven-point hedonic scale ranging from "Liked Very Much" over "Neither Liked Nor Disliked" to "Disliked Very Much." The duration of this study will be one hour.

I hereby acknowledge that the following points concerning this study have been thoroughly explained to me:

1. Dr. Elie S. Nuwayser, Biotek, Inc., 21-C Olympia Avenue, Woburn, MA, 01801, (617) 938-0938 is the Principal Investigator in this study who may be contacted.

2. Accidental exposure of the repellent product to the eyes or mucous membranes should be avoided because the product can be an irritant to those areas.

3. I have been given the opportunity to ask all questions regarding the study and they have been answered to my satisfaction.

4. I do understand that I must keep my scheduled appointments, since this is important for the success of the study. My failure to do so will result in my disqualification as a test subject.

Herbert V. Shuster Inc
Quincy Research Park
5 Hayward Street
Quincy, Massachusetts 02171
Telephone 617 328-7600

BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

5. I will follow the specific directions given to me and will not willfully do anything that might change the results of the study.

6. If I have any questions during the study, I should contact Dr. Kurt S. Konigsbacher, Vice President, Corporate Development, at (617) 328-7600.

7. If I am injured as a direct result of administration of the test materials., I understand that medical treatment shall be made available through Herbert V. Shuster, Inc. Financial compensation for such things as lost wages, disability or discomfort due to the type of injury is not available. I understand, however, that I have not waived any of my legal rights by signing this form.

8. I understand that participation in this study is voluntary and I may withdraw from the study at any time. If I am dismissed from the study for medical reasons (such as inability to tolerate the test products or procedures), my compensation will be proportional to the time spent in the study. If I am dismissed from the study because I have not complied with the protocol or withdraw from the study without valid reason, I shall receive no compensation at all.

As a volunteer participating in the study outlined above, I acknowledge and certify that:

1. I am aware that the United States Army Medical Research and Development Command is sponsoring the study.

2. I am aware of the nature, purpose and duration of the study and that the foreseeable health risks of any participation in the study are limited to the adverse reactions of the products being tested. In addition, there may be other risks which are unforeseeable.

3. I am aware that I will not directly receive health benefits from the study.

4. I am aware that copies of all data obtained during the course of this study are the property of the sponsor. My name will be disclosed to the sponsor. I am also aware that if this study is ever subject to inspection by the Food and Drug Administration or other government agencies, the government investigators may review the data as well as obtain copies of it and/or this informed consent. I understand that all this information is considered by Herbert V. Shuster, Inc., as strictly confidential.

BIOTEK, INC.

SUSTAINED RELEASE MOSQUITO REPELLENT

SUBJECT INFORMED CONSENT

5. I am aware that any questions I might have concerning the procedures which affect me will be answered promptly and completely to my satisfaction by the Principal Investigator or by Dr. Kurt S. Konigsbacher.

6. I am aware that I have the right to withdraw my consent and discontinue my participation in this study at any time.

7. I am aware that this study involves a total of twenty-five (25) subjects for evaluation of how well the product is liked.

8. I have the legal capacity to sign this informed consent.

9. I am aware that I will receive a copy of this informed consent.

Executed this _____ (day) day of _____ (month), 1985.

Subject Name: _____

Address: _____

City: _____, Zip _____

Phone No.: _____

Birthdate: _____

Identity Verification: _____

Soc.Sec. No.: _____

Witnessed by: _____ (Staff Member)

Figure 5

MOSQUITO REPELLENT QUESTIONNAIRE

Name: _____ Date: _____

	Code	
1. How well do you like this product?	_____	_____
(7) Like very much	_____	_____
(6) Like moderately	_____	_____
(5) Like slightly	_____	_____
(4) Neither like nor dislike	_____	_____
(3) Dislike slightly	_____	_____
(2) Dislike moderately	_____	_____
(1) Dislike very much	_____	_____

2. Please give reasons for your score: _____

_____

_____

_____

3. How does this product feel on your skin?

(5) Excellent	_____	_____
(4) Very good	_____	_____
(3) Good	_____	_____
(2) Fair	_____	_____
(1) Poor	_____	_____

4. How strong is the odor level after application?

(5) Very strong	_____	_____
(4) Strong	_____	_____
(3) Moderate	_____	_____
(2) Slight	_____	_____
(1) None	_____	_____

5. Do you have any comments or suggestions? _____

_____

_____

_____

APPENDIX III

MILITARY MATERIALS COMPATIBILITY TESTING PROTOCOLS

TEST PROCEDURE NO. P-1

PLASTICS TEST

Procedure

1. Cut three samples of each plastic (approximately 1" x 1") and label A, B, and C.
2. Smooth all edges using zero or finer grade sandpaper or emery cloth.
3. Accurately measure the length, width, and thickness of each sample using a micrometer.
4. Accurately weigh each sample using an analytical sample.
5. Place 10 ml of the alcoholic lotion base in a clean glass beaker and totally submerge sample A for 24 hours. (Note: If sample floats, secure it with a piece of nichrome wire.) Cover beaker with a watchglass to minimize evaporation and note time of immersion.
6. Place 10 ml of the current Army DEET formulation in a clean glass beaker and totally submerge sample B for 24 hours. Cover beaker with a watchglass to minimize evaporation and note the time of immersion.
7. Place 10 ml of the time release DEET formulation in a clean glass beaker and totally submerge sample C for 24 hours. Cover beaker with a watchglass to minimize evaporation and note the time of immersion.
8. Using forceps, individually remove each sample from solution and weigh accurately.
9. Carefully wash each sample in soapy water, flush with distilled water and dry in a vacuum chamber.
10. After each sample is completely dry, measure all dimensions and weigh the sample carefully.
11. Subject all samples to the following tests:

    A. Gloss Loss: Visually compare sample A, B, and C and note loss of luster.

    B. Decomposition: Visually compare sample A, B, and C and note any decomposition or film formation.

    C. Discoloration: Visually compare sample A, B, and C and note any change in color.

    D. Swelling: Compare the dimensions of each sample before and after immersion.

    E. Clouding: Compare samples A, B, and C under a 7X magnifying glass or stereomicroscope and note any changes.

F. Crazing:

G. Bubbling:

H. Cracking:

Compare samples A, B, and C under a 7X magnifying glass or stereomicroscope and note any changes.

I. Solubility: Note if sample dissolves.

J. Tackiness: Clamp gently with tweezers and release. Note change in texture.

TEST PROCEDURE NO. AD-1

ADHESIVES TEST

(Surgical Tape)

1. Label three surgical tape samples (3" x 1/2") A, B, and C.
2. Carefully adhere each sample to a clean ceramic plate so that a 1/2 inch piece at the end of the strip is perpendicular to the plate.
3. Place a measured amount of the alcoholic lotion base, the current Army DEET formulation, and the time release DEET formulation on samples A, B, and C respectively, and spread evenly with a glass rod over a 1" x 1" area.
4. Allow to stand for 24 hours.
5. Using a Haldex tension gauge in conjunction with a constant speed motor, clamp and pulley, measure the force required to pull the adhesive strips free of the porcelain.
6. Record and compare results obtained in A, B, and C.
7. Repeat 4X and use the mean values to calculate any loss of adhesion.

TEST PROCEDURE NO. AD-2

ADHESIVES TEST

(Adhesive Bandage)

1. Label three adhesive bandages on the back of the gauze as A, B, and C.
2. Remove backing carefully and stick to a clean ceramic plate so that the gauze area is perpendicular to the adhesive area.
3. Place measured amount of the alcoholic lotion base, the current Army DEET formulation, and the time release DEET formulation on samples A, B, and C respectively, and carefully spread evenly with a glass rod over each of the 1" x 1" areas.
4. Allow to stand for 24 hours.
5. Using a Haldex tension gauge in conjunction with a constant speed motor, clamp and pulley, measure the force required to pull one of the adhesive strips free from the ceramic.
6. Record and compare results obtained in A, B, and C.
7. Repeat 4X and use the mean values to calculate any loss or gain of adhesion.

TEST PROCEDURE NO. RE-1

RUBBER AND ELASTOMER TEST

Procedure

1. Cut three samples of each type of rubber or elastomer (approximately 1 1/2" x 3") and label A, B, and C.

2. Carefully measure the length, width, and thickness of each of the samples using a micrometer.

3. Accurately weigh each sample using an analytical balance.

4. Fasten each sample to a wooden board (15" x 9") using aluminum tacks. (Failure to use aluminum may result in cracking of the sample due to ozone.)

5. Using a suitable pipette, place a measured amount of the alcoholic lotion base, the current Army DEET formulation and the time release DEET formulation on samples A, B, and C, respectively.

6. Spread the solutions evenly using a clean glass rod over a 3" x 1" area.

7. After 24 hours, visually inspect the samples for cracking, deterioration, or any other unusual characteristics, using a 7X magnifying glass or stereomicroscope.

8. Carefully wash all samples in mild, soapy water, flush thoroughly with distilled water, and dry in a vacuum chamber.

9. Weigh each sample accurately and measure the thickness of each sample for any evidence of swelling.

TEST PROCEDURE NO. O-1

ORGANIC COATINGS TEST

Procedure

1. Place the coated panel to be tested on a firm, horizontal surface.
2. Each coated panel shall be separated into three (1" x 1") areas and labelled A, B, and C.
3. Place a measured amount of the alcoholic lotion base, the current Army DEET formulation, and the time release DEET formulation on areas A, B, and C, respectively.
4. Spread each solution evenly using clean glass rods.
5. After 24 hours, carefully wash all areas with mild, soapy water, flush thoroughly with distilled water and dry in a vacuum chamber.
6. Conduct the ASTM Film Hardness Test (D-3363-74) and compare the results obtained in samples A, B, and C.
7. Record any evidence of bubbling, cracking, and/or visible deterioration using a 7X magnifying glass or stereomicroscope.

TEST PROCEDURE NO. O-2

ACRYLIC CAMOUFLAGE PAINT TEST

Procedure

1. Smear each color of camouflage paint on a clean glass slide so that visibility is not possible through a 1" x 1" portion of the slide.

2. Place each slide on a 20 x 20 per inch graph paper and count the number of squares covered by the camouflage paint (380-400 squares must be covered).

3. Secure the glass slide to each of the three blades of a low sheer (zero pitch) impeller, allowing the 1" x 1" area with camouflage paint to extend one inch below the bottom of the blade.

4. Connect the impeller to a controlled speed motor set at 10 RPM.

5. Place 300 mls of the current Army DEET formulation in a 500 ml beaker and place the impeller in the solution so that the 1" x 1" area with camouflage paint is completely submerged and allow to rotate for 24 hours.

6. Repeat experiment with new slides, new samples, and the time release DEET formulation and the alcoholic lotion base.

7. Compare all three sets of slides, check the color of the solutions, and determine the visibility through the slides by placing each of the slides on 20 x 20 per inch graph paper and count the number of squares visible through the camouflage.

8. To determine the percent of camouflage paint lost, divide the total number of squares visible through the camouflage paint after immersion, by the total number of squares covered by the camouflage paint before immersion, and multiply by 100.

TEST PROCEDURE NO. M-1

METALS TEST

(Aluminum)

Procedure

1. Carefully cut three (1" x 1") squares of aluminum foil and label A, B, and C.

2. Accurately weigh each sample using an analytical balance.

3. Place 10 ml of the alcoholic lotion base in a clean glass beaker and totally submerge sample A for 24 hours. Note time of immersion. (Cover beaker with watchglass to minimize evaporation).

4. Place 10 ml of the current Army DEET formulation in a clean glass beaker and totally submerge sample B for 24 hours. Note time of immersion. (Cover beaker with watchglass to minimize evaporation.)

5. Place 10 ml of the Time Release DEET formulation in a clean glass beaker and totally submerge sample C for 24 hours. Note time of immersion. (Cover beaker with watchglass to minimize evaporation.)

6. Using forceps, individually remove each sample from solution and weigh accurately in a weighing vessel.

7. Carefully wash each sample in soapy water, flush with distilled water, and dry in a vacuum chamber.

8. Re-weigh both samples accurately and compare the results.

9. Visually inspect each sample using a 7X stereomicroscope or magnifying glass for any visible corrosion, pitting, and gloss loss.

TEST PROCEDURE N-1

LEATHER TEST

Procedure

1. Cut three samples of leather (approximately 1" x 1") and label A, B, and C.

2. Place 10 ml of the alcoholic lotion base in a clean glass beaker and totally submerge sample A for 24 hours. (Note: If sample floats, secure it with a piece of nichrome wire.) Cover beaker with a watchglass to minimize evaporation and note time of immersion.

3. Place 10 ml of the current Army deet formulation in a clean glass beaker and totally submerge sample B for 24 hours. Cover beaker with a watchglass to minimize evaporation and note the time of immersion.

4. Place 10 ml of the time release deet formulation in a clean glass beaker and totally submerge sample C for 24 hours. Cover beaker with a watchglass to minimize evaporation and note the time of immersion.

5. Using forceps, individually remove each sample from solution and view with a 7X stereomicroscope.

6. Wash samples with soapy water and rinse with distilled water and vacuum dry.

7. Note any visible changes using a 7X stereomicroscope or any deterioration in the surface of the leather sample.

TEST PROCEDURE NO. T-1

TEXTILES TEST

1. Cut three strips, 1" x 6", from each fabric to be tested and label as A, B, and C.
2. Immerse samples A, B, and C in the alcoholic lotion base, the current Army Deet formulation and the time release DEET formulation respectively, for 24 hours.
3. Remove samples A, B, and C and vacuum dry.
4. Send all samples to a laboratory capable of measuring the breaking load and elongation of textile fabrics according to ASTM (D1682-64) protocol.
5. Compare measurements and determine if the breaking load and elongation of the material is affected by DEET.

APPENDIX IV

MILITARY MATERIALS COMPATIBILITY TESTING RESULTS

Test Sample: Current Army Deet Formulation

Exposure Time: 24 Hours

Test Procedure Number	Material	Test Result
	PLASTICS	
P-1	Cellulose Acetate Butyrate	Totally solvated.
P-1	Low Density Polyethylene	Slight gloss loss, no other visible changes.
P-1	Methyl Methacrylate	Slight gloss loss, no other visible changes.
P-1	Nylon 6/6	No visible changes.
P-1	Polyacrylamide (Lucite)	Extreme gloss loss, clouded, swelling evident (5% increase), slight solubility.
P-1	Polycarbonate (Lexan)	Slight clouding.
P-1	Polyurethane	Moderate gloss loss, slightly discolored, swelling evident (15% increase), definite tackiness.
	ADHESIVES	
AD-1	Surgical Tape	881.25 gm of force required to remove surgical tape sample.
AD-2	Adhesive Bandages	1468.75 gm of force required to remove adhesive bandage sample.
	RUBBER AND ELASTOMERS	
RE-1	Latex Rubber	No visible changes.
RE-2	Silicone Rubber	No visible changes.
	ORGANIC COATINGS	
O-1	Auto Enamel	No visible changes. Scratch and gouge hardness = 4H.
O-2	Acrylic Camouflage Paint	88.2% loss of dark green camouflage,

		14.7% loss of white camouflage, 30.6% loss of light green camouflage.
O-3	Polyurethane	Scratch and gouge hardness less than 6B. Definite deterioration and bubbling.
	METALS	
M-1	Aluminum	No visible changes.
	NATURAL PRODUCTS	
N-1	Leather	No visible changes.
	TEXTILES	
T-1	Cotton (100%)	No visible changes.
T-1	Cellulose Acetate (Rayon)	No visible changes.
T-1	Cotton-Nylon (50/50)	No visible changes in fiber; dark green dye is slightly leached.
T-1	Polyester	No visible changes.

Test Sample: Alcoholic Lotion Base

Exposure Time: 24 Hours

Test Procedure Number	Material	Test Result
	PLASTICS	
P-1	Cellulose Acetate Butyrate	No visible changes.
P-1	Low Density Polyethylene	No visible changes.
P-1	Methyl Methacrylate	No visible changes.
P-1	Nylon 6/6	No visible changes.
P-1	Polyacrylamide (Lucite)	No visible changes.
P-1	Polycarbonate (Lexan)	No visible changes.
P-1	Polyurethane	No visible changes.
	ADHESIVES	
AD-1	Surgical Tape	700.0 gm of force required to remove surgical tape sample.
AD-2	Adhesive Bandages	1498.75 gm of force required to remove adhesive bandage sample.
	RUBBER AND ELASTOMERS	
RE-1	Latex Rubber	No visible changes.
RE-2	Silicone Rubber	No visible changes.
	ORGANIC COATINGS	
O-1	Auto Enamel	No visible changes. Scratch and gouge hardness = 4H.
O-2	Acrylic Camouflage Paint	97.1% loss of dark green camouflage, 98.2% loss of white camouflage, 99.7% loss of light green camouflage.
O-3	Polyurethane	Scratch and gouge hardness = 5B. Definite gloss loss, no bubbling, tacky to touch.

	METALS	
M-1	Aluminum	No visible changes.
	NATURAL PRODUCTS	
N-1	Leather	No visible changes.
	TEXTILES	
T-1	Cotton (100%)	No visible changes.
T-1	Cellulose Acetate (Rayon)	No visible changes.
T-1	Cotton-Nylon (50/50)	No visible changes in fibers; leaching of dark green dye.
T-1	Polyester	No visible changes.

Test Sample: Time Release DEET Formulation (HSL-44)

Exposure Time: 24 Hours

Test-Procedure Number	Material	Test Result
	PLASTICS	
P-1	Cellulose Acetate Butyrate	Totally solvated.
P-1	Low Density Polyethylene	No visible changes.
P-1	Methyl Methacrylate	No visible changes.
P-1	Nylon 6/6	No visible changes.
P-1	Polyacrylamide (Lucite)	Slight gloss loss; greyish film forms; slight clouding.
P-1	Polycarbonate (Lexan)	Slight clouding; greyish film forms.
P-1	Polyurethane	Slightly tacky to touch; definite swelling (17% increase in thickness); slight swelling.
	ADHESIVES	
AD-1	Surgical Tape	672.50 gm of force required to remove surgical tape sample.
AD-2	Adhesive Bandages	1493.75 gm of force required to remove adhesive bandage sample.
	RUBBER AND ELASTOMERS	
RE-1	Latex Rubber	No visible changes.
RE-2	Silicone Rubber	No visible changes.
	ORGANIC COATINGS	
O-1	Auto Enamel	No visible changes. Scratch and gouge hardness = 4H.
O-2	Acrylic Camouflage Paint	74.2% loss of dark green camouflage, 10.5% loss of white camouflage, 34.7% loss of light green camouflage.

O-3	Polyurethane	Gouge hardness = 5B; Scratch hardness = 4B.
	METALS	
M-1	Aluminum	No visible changes.
	NATURAL PRODUCTS	
N-1	Leather	No visible changes.
	TEXTILES	
T-1	Cotton (100%)	No visible changes.
T-1	Cellulose Acetate (Rayon)	No visible changes.
T-1	Cotton-Nylon (50/50)	No visible changes in fibers; slight leaching of dark green dye.
T-1	Polyester	No visible changes.

DISTRIBUTION LIST

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